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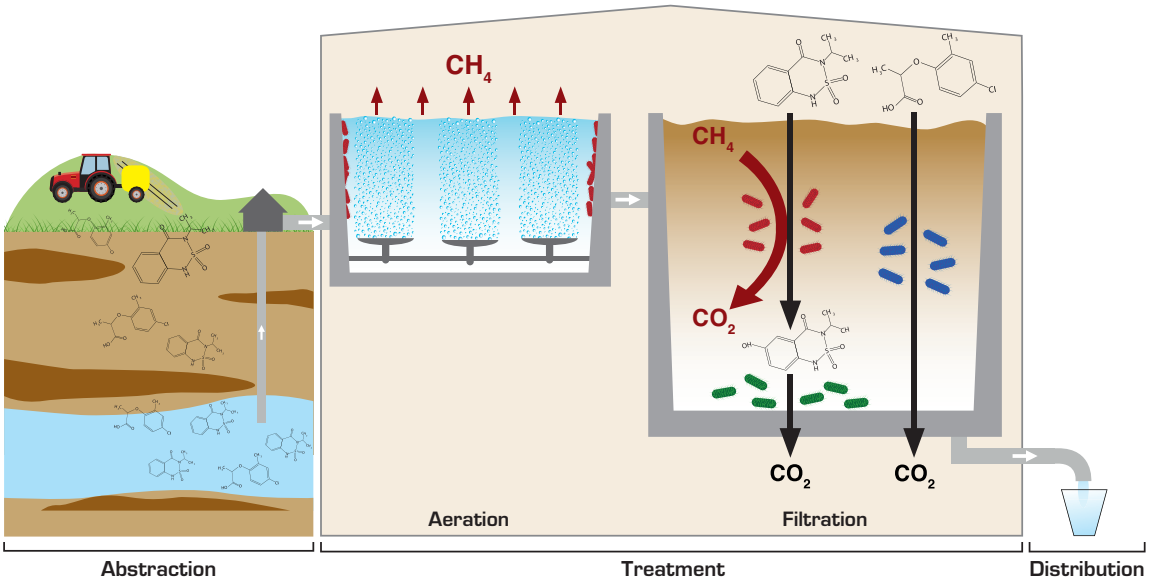

Mathilde J. Hedegaard

PhD Thesis
February 2018

DTU Environment
Department of Environmental Engineering

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Preface

This PhD thesis is based on research carried out at Department of Environmental Engineering, Technical University of Denmark (DTU). The project was conducted in the period May 2013 to November 2017 (including a maternity leave and 9 months leave during which other research projects were carried out). The PhD project was supervised by Prof. Hans-Jørgen Albrechtsen (main supervisor), DTU Environment, and PhD Rasmus Boe-Hansen (co-supervisor), Krüger Veolia A/S.

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

I **Hedegaard, M. J.**, Arvin, E., Corfitzen, C. B., Albrechtsen, H.-J., 2014. Mecoprop (MCPP) removal in full-scale rapid sand filters at a groundwater-based waterworks. *Science of the Total Environment*, Vol. 499, pp. 257-264.

II **Hedegaard, M. J.**, Deliniere, H., Prasse, C., Dechesne, A., Smets, B. F., Albrechtsen, H.-J., 2018. Evidence of co-metabolic bentazone transformation by methanotrophic enrichment from a groundwater-fed rapid sand filter. *Water Research*, Vol. 129, pp 105-114.

III **Hedegaard, M. J.,** Prasse, C., Albrechtsen, H.-J. Degradation pathways of the herbicide bentazone in filter sand used for drinking water treatment. *Submitted*

IV Papadopoulou, A., **Hedegaard, M. J.,** Dechesne, A., Albrechtsen, H.-J., Musovic, S., Smets, B. F. Methanotrophic contribution to phenoxy acids degradation in cultures enriched from a groundwater-fed rapid sand filter. *Manuscript*

V **Hedegaard, M. J.**, Sykyta, M. A. M., Milanovic, N., Lee, C. O., Boe-Hansen, R., Albrechtsen, H.-J. Importance of methane oxidation for microbial degradation potential of the herbicide bentazone in drinking water production. *Draft manuscript*

In this online version of the thesis, paper **I-V** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

In addition, the following publications, not included in this thesis, were also conducted during this PhD study:

Papers:

Hedegaard, MJ & Albrechtsen, H-J 2017, 'Corrigendum to "Microbial pesticide removal in rapid sand filters for drinking water treatment - Potential and kinetics" [Water Res. 48 (2014) 71-81]' *Water Research*, vol 122, pp. 708-713. DOI: 10.1016/j.watres.2017.05.071

Hedegaard, MJ & Albrechtsen, H-J 2014, 'Fjernelse af pesticider i sandfiltre' *DanskVand*, no. 6, pp. 58-59.

Hedegaard, MJ & Albrechtsen, H-J 2014, 'Microbial pesticide removal in rapid sand filters for drinking water treatment – Potential and kinetics' *Water Research*, vol 48, pp. 71-81. DOI: 10.1016/j.watres.2013.09.024

Conference proceedings

Lee, CO, Musovic, S, Hedegaard, MJ, Tatari, K, Laugesen, H & Albrechtsen, H 2017 'Pesticide degradation potential of pesticides in biological rapid sand filters at 10 different waterworks' in *American Water Works Association Water Quality and Technology Conference* , Portland, Oregon, USA, 12/11/2017-16/11/2017.

Hedegaard, MJ, Delinere, H, Prasse, C, Dechesne, A, Smets, BF & Albrechtsen, H-J 2016, 'Sammenhæng mellem aktivitet af metanoksiderende bakterier, opformeret fra sandfiltre på danske vandværker, og nedbrydningen af pesticidet bentazon' *Dansk Vand konference*, Aarhus, Denmark, 08/11/2016-09/11/2016.

Hedegaard, MJ & Albrechtsen, H-J 2015, 'Removal of pesticides with filter sand from biological rapid sand filters' in *IWA Specialized Conference Biofilms in drinking water systems from treatment to tap.* IWA Publishing Company, Arosa, Switzerland, pp. 236-237.

Hedegaard, MJ & Albrechtsen, H-J 2015 'Removal of pesticides with filter sand from rapid sand filters at Danish waterworks' *9th annual DWF meeting*, Frederiksberg, Denmark, 29/01/2015.

Papadopoulou, A, Hedegaard, MJ, Dechesne, A, Albrechtsen, H-J & Smets, BF 2014, 'Methanotrophs assisted bentazone *degradation' International Biodegradation and Biodeterioration Symposium*, Lodz, Poland, 03/09/2014 - 05/09/2014,

Hedegaard, MJ & Albrechtsen, H-J 2014, 'Microbial pesticide degradation processes in rapid sand filters for treatment of groundwater for drinking water production' *AWWA Water Quality Technology Conference & Exposition,* New Orleans, LA, United States, 16/11/2014 - 20/11/2014,

Hedegaard, MJ & Albrechtsen, H-J 2014, 'Pesticidnedbrydning' *Temadag om biologiske sandfiltre til drikkevandsbehandling,* DANVA, Skanderborg, Denmark, 22/01/2014

Hedegaard, MJ & Albrechtsen, H-J 2014, 'Pesticide degradation in rapid sand filters' *Nordic Water Conference 2014* , Helsinki, Finland, 02/06/2014 - 04/06/2014,

Hedegaard, MJ & Albrechtsen, H-J 2014, 'Processes of microbial pesticide degradation in rapid sand filters for treatment of drinking water' *2014 IWA World Water Congress & Exhibition*, Lisbon, Portugal, 21/09/2014 - 26/09/2014,

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I am also grateful to my family and friends, especially my parents for their life long support to pursue my goals. Most importantly, I would like to thank my husband, Kim, and daughters, Alberte and Nanna, for their support through good times and bad, and for every day reminding me of the most important things in life.

Summary

Groundwater is used as drinking water source all over the world. However, large parts are contaminated by pesticides at low concentrations (sub µg/L), due to anthropogenic activities. These pesticides can adversely impact human health, and have legal implications. Thus, it is important to identify sustainable methods to remove pesticides from polluted water sources. Aeration of anaerobic groundwater, followed by biological rapid sand filtration is a widespread technology in drinking water treatment. Even though these systems are not designed for removal of trace contaminants, they have shown potential for microbial degradation of pesticides and their degradation products. If pesticides can be removed in rapid sand filters, it is of large commercial interest due to the importance in maintaining a simple, sustainable water treatment. To take advantage of the microbial pesticide degradation and identify associated risks, it is necessary to understand the extent of pesticide degradation and the governing microbial processes in the water treatment.

The objective of this PhD thesis was to investigate the potential for microbial pesticide degradation at waterworks treating groundwater and to investigate, which microbial processes govern the degradation, in order to suggest how pesticide degradation can be stimulated in water treatment systems.

In a full-scale waterworks the rapid sand filters removed a phenoxy acid (herbicide) contamination from drinking water and investigations showed a potential for removing several pesticides in filter sand from different waterworks. The largest biological pesticide removal was observed in filter sand from a waterworks characterised by methane-rich groundwater. Thus, it was investigated for a connection between pesticide degradation and methane oxidation.

In an enrichment culture, methanotrophs contributed to the degradation of phenoxy acids. However, a phenoxy acid was degraded in filter sand from 10 different waterworks receiving groundwater with varying concentrations and absence of methane. The omnipresent phenoxy acid degradation demonstrated, that degradation in rapid sand filters was not associated with methane oxidation. Based on the present investigations and literature, it was suggested that phenoxy acid degradation in rapid sand filters is due to primary metabolism, and that degradation might be stimulated by enriching naturally occurring specific degraders in the sand filters upon exposure to phenoxy acid contaminated groundwater.

A suite of evidence showed that the herbicide bentazone was co-metabolically transformed to hydroxy-bentazone by the methanotrophic enrichment culture. Subsequently, it was investigated whether bentazone degradation was also connected with methane oxidation in drinking water treatment systems.

In waterworks wells in Denmark, bentazone was detected significantly less frequently in wells with methane than in wells without methane. Similarly, the biological bentazone removal in filter sand from 14 waterworks correlated significantly with the maximum methane concentration in the raw water and did not correlate with other water quality parameters, such as the ammonium concentration. Furthermore, the connection between bentazone degradation and methane oxidation in filter sand was demonstrated by inhibition experiments, in which acetylene inhibited *both* the methane oxidation and the bentazone removal. The main biotransformation pathways clearly showed the importance of initial hydroxylation reactions during bentazone degradation in filter sand. However, bentazone was further degraded in filter sand and showed that not only methanotrophs, but also other heterotrophs contributed to the degradation. Methanotrophic biomass from the aeration tanks clearly demonstrated a bentazone degradation, which depended on the presence of methane.

Transformation yields describing the bentazone removal versus the methane oxidation were in same order of magnitude for all investigated media: methanotrophic enrichment cultures, filter sand and biomass from aeration tanks, which strongly indicated that the same degradation process governed bentazone removal in the different media. It was suggested that full-scale waterworks operates like a sequential reactor system, where methanotrophs are grown in the aeration tanks and transported to the rapid sand filters where they can perform co-metabolic pesticide biodegradation. It was suggested that bentazone removal can be stimulated at waterworks, by stimulating growth of methanotrophs.

Overall, this PhD demonstrated a substantial potential for biological pesticide degradation in drinking water treatment systems. While the omnipresent phenoxy acid degradation potential was probably due to specific degraders, bentazone degradation was connected with the methane oxidation. Based on the present investigations and literature, it was suggested that phenoxy acid degradation can be stimulated by enrichment of naturally occurring degraders in filter sand, and that bentazone degradation can be stimulated by stimulating growth of methanotrophs in the water treatment.

Dansk sammenfatning

Grundvand anvendes som drikkevandsressource over hele verden. Menneskeskabte pesticidforureninger er dog desværre et problem for anvendelsen af store dele af grundvandet, da disse kan have skadelige effekter på mennesker og kan medføre overskridelser af lovgivningen. Det er derfor vigtigt at identificere bæredygtige vandbehandlingsløsninger, som kan fjerne lave koncentrationer af pesticider (µg/L). Biologisk vandbehandling er en udbredt proces, som består af beluftning af anaerobt grundvand, efterfulgt af filtrering i sandfiltre. Selvom denne vandbehandlingsproces ikke er designet til at håndtere pesticidforureninger, har den udvist et potentiale for pesticid fjernelse. Det vil have kommerciel interesse at fjerne pesticider i den eksisterende vandbehandling, da denne er både økonomisk og miljømæssig bæredygtig. For at kunne udnytte pesticidnedbrydning i vandbehandlingen og kende de dertilhørende ricisi er det dog nødvendigt at forstå udbredelsen af pesticidnedbrydning samt de styrende nedbrydningsprocesser.

Formålet med denne PhD var at undersøge potentialet for biologisk pesticidnedbrydning på vandværker, der behandler grundvand, og undersøge hvilke biologiske strategier, der var styrende for pesticidnedbrydningen, med henblik på at undersøge hvordan pesticidnedbrydning kan igangsættes eller stimuleres i biologiske vandbehandlingssystemer.

På et eksisterende vandværk blev en forurening med en phenoxysyre (ukrudtsmiddel) fjernet fra drikkevandet i sandfiltrene. Efterfølgende undersøgelser viste, at der var et potentiale for at fjerne flere forskellige pesticider i sand fra filtre på forskellige vandværker. Den største biologiske fjernelse blev observeret i sand fra et vandværk, der var karakteriseret af høje koncentrationer af metan i råvandet. Det blev derfor undersøgt, om pesticidfjernelsen kunne forbindes med metan oxidation.

I en berigelseskultur medvirkede de metanotrofe bakterier til nedbrydningen af phenoxysyrer. Imidlertid viste den allestedsnærværende fjernelse af en phenoxysyre i filtersand, fra 10 vandværker med varierende koncentrationer af metan i råvandet, at nedbrydningen af phenoxysyre i filtersand ikke kunne tilskrives metanotrofe bakterier. Derimod indikerede disse resultater, at phenoxysyrer blev nedbrudt ved primær metabolisme. Baseret på disse resulter og resultater fra litteraturen, blev det foreslået, at nedbrydningen af phenoxysyrer kan stimuleres ved at eksponere sandfiltrene for

phenoxysyreforurenet grundvand og dermed opdyrke naturligt forekommende bakterier.

Ukrudtsmidlet bentazon blev co-metabolsk nedbrudt til hydroxy-bentazon af den metanotrofe berigelseskultur. Det blev derfor undersøgt, om bentazon også kunne nedbrydes af metanotrofe bakterier på vandværker.

Bentazon blev fundet signifikant sjældnere i vandværksboringer med metan end i boringer uden metan i Danmark. På samme måde hang den biologiske fjernelse af bentazon i filtersand fra 14 vandværker signifikant sammen med koncentrationen af metan i råvandet, imens der ikke var nogen sammenhæng med andre vandkvalitetsparametre, såsom ammonium koncentrationen. Den metanotrofe co-metabolske bentazonnedbrydning i filtersand blev ydermere påvist ved hæmningsforsøg, hvor tilsætning af acetylene stoppede *både* metan oxidationen og fjernelsen af bentazon. Kortlægningen af de primære nedbrydningsveje for bentazon i filtersand påviste, at hydroxyleringsreaktioner var vigtige for nedbrydningen. Nedbrydningsvejene viste desuden, at ikke kun metanotrofe bakterier men også andre mikroorganismer bidrog til nedbrydningen af bentazon i filtersandet. Metanotrof-biomasse fra beluftningstankene på et vandværk nedbrød bentazon co-metabolsk, og denne nedbrydning var afhængig af metans tilstedeværelse.

Forholdet mellem omdannelsen af bentazon og forbruget af metan var i samme størrelsesorden for alle de undersøgte medier: den metanotrofe berigelseskultur, filtersandet og biomasse fra beluftningstankene. Dette viste, at den samme biologiske proces var styrende for nedbrydningen af bentazon i alle de undersøgte medier. Det blev foreslået, at vandværker fungerer som et sekventiel reaktor system, hvor metanotrofe bakterier vokser i beluftningstankene og bliver transporteret med vandet til sandfiltrene, hvor de kan udføre co-metabolsk nedbrydning. Dermed blev det foreslået, at bentazon kan nedbrydes i biologisk vandbehandling ved at stimulere vækst af metanotrofe bakterier i denne proces.

Alt i alt er der i denne afhandling påvist et betydeligt potentiale for biologisk nedbrydning af pesticider i biologiske vandbehandlingssystemer. Phenoxysyre nedbrydes formodentligt af specifikke bakterier, imens bentazon nedbrydningen kunne forbindes med metan oxidation. Baseret på disse resulter og resultater fra litteraturen, blev det foreslået at nedbrydning af phenoxysyrer i sandfiltre kan stimuleres ved opdyrkning af naturligt forekommende bakterier, samt at bentazone kan fjernes ved at stimulere væksten af metanotrofe bakterier på vandværker.

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1 Introduction

Groundwater is used as drinking water source all over the world (IWA, 2014). Unfortunately, trace contaminants enter the groundwater from diffuse or point sources, such as use of pesticides in agriculture or urban areas. Pesticides are applied directly on land and can subsequently become mobile during rain events, where seepage and leaching processes allow the pesticides to infiltrate into the groundwater (Malaguerra et al., 2013). Semi-polar and polar pesticides can be especially mobile in aquifers (Clausen et al., 2001) resulting in steady fluxes even at locations distant from the application site. Thus, pesticides are detected in groundwater all over the world (Benner et al., 2013). In Denmark, where almost 100% of the drinking water is abstracted from of groundwater (IWA, 2014), pesticides and their metabolites were detected in 28.5% of the active waterworks wells and 49.5% of all monitoring wells in the period 1990-2015 (GEUS & Danish Ministry of Energy Utilities and Climate, 2016a) (Figure 1).

Pesticides pose a potential risk to humans and can cause unwanted effects on the environment (Aktar et al., 2009). Thus, the European Union (EU) has established the 'Drinking Water Directive', 'Water Framework Directive' and the 'Groundwater Directive', according to which the concentration of pesticides in drinking water and groundwater should not exceed 0.1 µg/L for a single compound, or 0.5 µg/L for the sum of all pesticides (European Commission, 2006, 2000, 1998). In spite of this, pesticides were detected 87 times above the guideline value in the effluent from Danish waterworks since 2012 (Bredsdorff, 2017; Ministry of Environment and Food of Denmark, 2017a). Therefore sustainable methods to remove pesticides at low concentrations (sub µg/L) need to be identified urgently.

Pesticides and other pollutants can be removed from drinking water by treatment processes such as granular activated carbon (GAC) (e.g. Heijman et al. 2002) and advanced oxidation processes (e.g. Suty et al. 2004). Utilization of biologically active rapid sand filters was recently suggested as treatment technology for pesticide removal (Benner et al., 2013), since rapid sand filters have the potential to be a cheaper and more sustainable treatment than the above-mentioned processes.

Rapid sand filters are used as a widespread technology in drinking water treatment, as they allow physio-chemical as well as microbial removal of several compounds such as manganese, methane, ferrous iron, ammonium etc. from

the drinking water. Even though biological filters are not designed for biological removal of pharmaceuticals and pesticides, such a removal potential has been demonstrated (Hedegaard and Albrechtsen, 2014; Kaiser et al., 2014; Zearley and Summers, 2012). Pesticide degradation in rapid sand filters constitutes an unexploited treatment process, that might already occur at full-scale waterworks (Figure 1). To take advantage of microbial pesticide degradation in sand filters and identify associated risks, it is necessary to understand the degradation processes occurring in the filters. While thorough literature exists on pesticide degradation in environmental systems such as soils and aquifers, the literature on degradation of pesticides in rapid sand filters is limited. The general understanding is, that rapid sand filters are dominated by prokaryotes (Albers et al., 2015a; Gülay et al., 2016; Palomo et al., 2016), while for example degradation of the herbicide bentazone is primarily ascribed to fungi in soils (Huber and Otto, 1994). Additionally, aeration of anaerobic groundwater, prior to rapid sand filtration, changes the redox potential compared to the aquifers, and induce a diverse microbial community in the filter sand, which depends on the groundwater chemistry (Albers et al., 2015a; Gülay et al., 2016; Palomo et al., 2016). Pesticide degradation in rapid sand filters is therefore likely to deviate from the current knowledge regarding degradation in soils and aquifers.

Hence, it is not yet known to which extent microbial pesticide degradation occurs in rapid sand filters, and the governing microbial degradation processes are also unknown.

1.1 Objectives and motivation

The overall objective of this PhD thesis was to investigate the potential for microbial pesticide degradation at waterworks treating groundwater, and to investigate which microbial processes govern pesticide degradation, in order to determine how pesticide degradation can be initiated or stimulated in biological water treatment systems. Of particular interest was the association between methane oxidation and the degradation of phenoxy acids and bentazone (Figure 1). The primary aims, which were addressed in this thesis were;

1. Investigate pesticide degradation at waterworks treating groundwater, and the degradation potential in filter sand from rapid sand filters.
2. Investigate whether primary metabolism and/or co-metabolism govern microbial pesticide degradation processes in rapid sand filters.
3. Suggest how pesticide degradation can be stimulated in biological drinking water treatment.

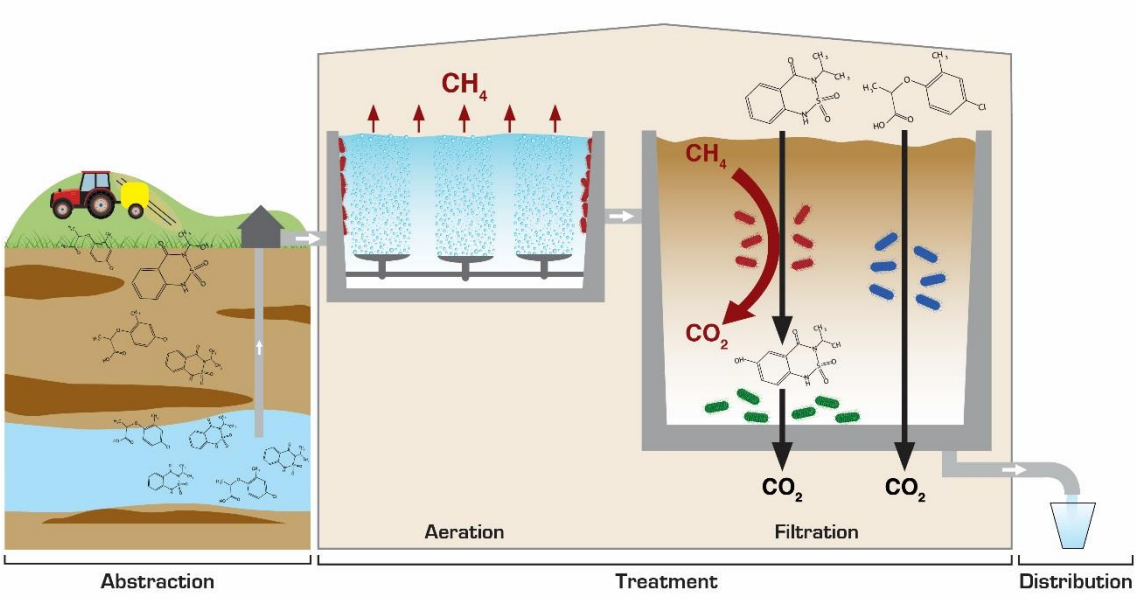


Figure 1 Abstraction of pesticide contaminated groundwater and microbial degradation in the water treatment prior to distribution to the consumers. The aims of this PhD thesis were to investigate microbial pesticide degradation potential in filter sand from rapid sand filters. To investigate whether primary metabolism (blue microbial community) and/or co-metabolism (red microbial community) with a subsequent utilization of oxidized transformation products by other heterotrophs (green microbial community) governed the degradation, and finally suggest how these processes can be stimulated in biological drinking water treatment.

2 Background

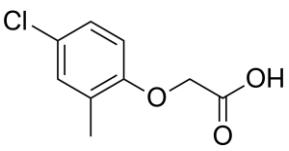
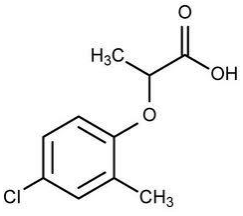
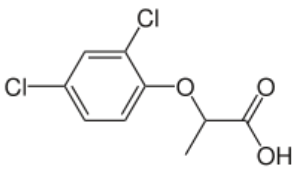
2.1 Pesticide contamination and degradation

For decades, pesticides have been used extensively all over the world to decrease unwanted organisms and competition from weeds and thereby increase crop yield. Thus they are detected in many fresh water bodies due to their environmental mobility and persistence. For example, in Denmark, pesticides were detected in 28.5% of the waterworks wells in the period 1990-2015, and the most frequently detected compounds were 1) 2,6-dichlorobenzamide (BAM), 2) bentazone, 3) mecoprop (MCPP) and 4) dichlorprop (GEUS & Danish Ministry of Energy Utilities and Climate, 2016a). Other studies have investigated the degradation of BAM in rapid sand filters (Albers et al., 2015b; Vandermaesen et al., 2016). Therefore, this thesis focused on the degradation of phenoxy acids and bentazone (herbicides) in biological water treatment systems for treatment of groundwater. This section serves as background information for the investigated herbicides and the investigated biological water treatment processes.

2.1.1 Phenoxy acids

Phenoxy acids were introduced in the 1940s and 1950s and have been used all over the world (Müller et al., 2004) to control broadleaf weeds in production of e.g. corn and wheat (Copping and Hewitt, 1998). Phenoxy acids, including MCPA, MCPP (mecoprop) and dichlorprop (Table 1), are synthetic auxins that mimic plant growth hormones (Copping and Hewitt, 1998).

Table 1 Chemical structure of three common phenoxy acids

	MCPA	MCPP	Dichlorprop
IUPAC	(4-Chloro-2-methylphenoxy)acetic acid	(RS)-2-(4-chloro-2-methylphenoxy)propanoic acid)	(R)-2-(2,4-dichlorophenoxy)propanoic acid
Structure			

Phenoxy acids are all structurally related (Table 1). They are weak organic acids and consist of an aromatic ring substituted by di-, trichloro or chloromethyl-groups, which is coupled to a propionic or acetic acid group by an ether bond (British Crop Protection Council, 2003).

Even though the use of phenoxy acids is regulated by the Danish Ministry of the Environment (GEUS & Danish Ministry of Energy Utilities and Climate, 2016a), MCPP was detected in 2.4% and dichlorprop in 2.1% of the Danish drinking water abstraction wells (both exceeding the guideline concentration 0.2% of the wells) (GEUS & Danish Ministry of Energy Utilities and Climate, 2016a).

Phenoxy acids can be degraded in the environment with typical half-lives in soils of less than 30 days (Bælum et al., 2008). Until now, the focus when studying the degradation of phenoxy acids has mainly been on primary metabolism (especially on degradation of 2,4-D ((2,4-Dichlorophenoxy)acetic acid)) and the degradation pathway encoded by *tfdA*-like genes (Mcgowan et al., 1998; Müller et al., 2004). In this pathway, 2,4-D degradation is initiated by removal of the acidic side-chain by an α-ketoglutarate-dependent dioxygenase which is encoded by the *tfdA* gene. Subsequently the 2,4-dichlorophenol is hydroxylated by the *tfdB* gene, the 3,5-dichlorocatechol ring is opened by the *tfdC* gene and the 2,4-dichloromuconate is converted to succinate for cell metabolism (Don et al., 1985; Müller et al., 2004; Smejkal et al., 2001).

However, other α-KG dioxygenases are able to convert the phenoxy acids into the corresponding phenol, and so degradation of (*RS*)-dichloprop and (*RS*)-MCPP can for instance be initiated by the genes *rdpA* and *sdpA* (Müller et al., 2004; Paulin et al., 2011). Paulin et al. (2011) found that all three target genes; *tfdA, rdpA* and *sdpA* were expressed in an indigenous soil microbial community when exposed to (*RS*)-dichloprop.

2.1.2 Bentazone

Bentazone is a broad-spectrum herbicide, which is used extensively all around the world, and is still legally used in the European Union (European Commission, 2017). In Denmark the use of bentazone was reduced from 93 tonnes in 1995 to 24 tonnes in 2015 (Ministry of Environment and Food of Denmark, 2017b). However, bentazone was detected in 3.3% of the samples from active waterworks abstraction wells in Denmark in the period 1992-2015 (exceeding the guideline value in 0.5%) (GEUS & Danish Ministry of Energy Utilities and Climate, 2016a). Furthermore, the bentazone concentration exceeded the guideline value 18 times in the effluent water from waterworks (2012-2017) (Bredsdorff, 2017; Ministry of Environment and Food of Denmark, 2017a). Bentazone is also frequently detected in other countries. Hence, bentazone was detected in 1% of investigated groundwater samples in

the Netherlands and in 2% of groundwater samples in the United States (Kolpin et al., 2000; Schipper et al., 2008).

In fresh field soils bentazone can be degraded by aerobic microbial processes with an average half-life time of 11 days. The main transformation products from bentazone degradation in soils are 6-OH-bentazone and 8-OH-bentazone, but 2-amino-N-propan-2-ylbenzamide (AIBA) has also been detected (Huber and Otto, 1994; Knauber et al., 2000). These intermediates are very reactive and can be incorporated into the organic soil matter (Huber and Otto, 1994). Recently, *N*-methyl-bentazone was reported as a more persistent transformation product from bentazone degradation in soils (European Food Safety Authority, 2015). Bentazone is mobile (Clausen et al., 2001) and hard to degrade in aquifer material (no degradation observed in periods of up to 371 days) (Albrechtsen et al., 2001; Broholm et al., 2001) and can therefore be transported from the application site to groundwater abstraction wells.

2.2 Drinking water treatment

In Denmark almost 100% of drinking water is supplied by groundwater (IWA, 2014). Natural and anthropogenic inorganic and organic compounds in abstracted groundwater may exceed the water quality guidelines, thus calling for treatment. Biological treatment of anaerobic groundwater is typically initiated with aeration, which serves to add oxygen (to a concentration of 8-10 mg/L), while volatile compounds such as methane and hydrogen sulphide are stripped off. Increased oxygen concentrations are necessary for the subsequent removal of iron, manganese and ammonium. Aeration is followed by rapid sand filtration, which is designed for a contact time between 7.5 and 20 minutes. In Denmark, no disinfection is included in the treatment process or during distribution (Winter et al., 2010) (Figure 2).

Biological rapid sand filters are used in drinking water treatment plants globally (Mouchet, 1992; Zearley and Summers, 2012). These filters constitute a highly complex system of several simultaneous removal mechanisms, whereby iron (Fe^{2+}) and manganese (Mn^{2+}) are removed by physico-chemical and biological oxidation processes as well as precipitation (Mouchet, 1992; Tekerlekopoulou et al., 2013), while ammonium is oxidized biologically into nitrite and then nitrate (Lytle et al., 2007). Besides removing inorganic components, different investigations have shown that biological filters can remove organic chemicals such as methyl *tert*-butyl ether (MTBE) (Arvin et al., 2004), 2-methylisoborneol (MIB) and geosmin (Ho et al., 2007).

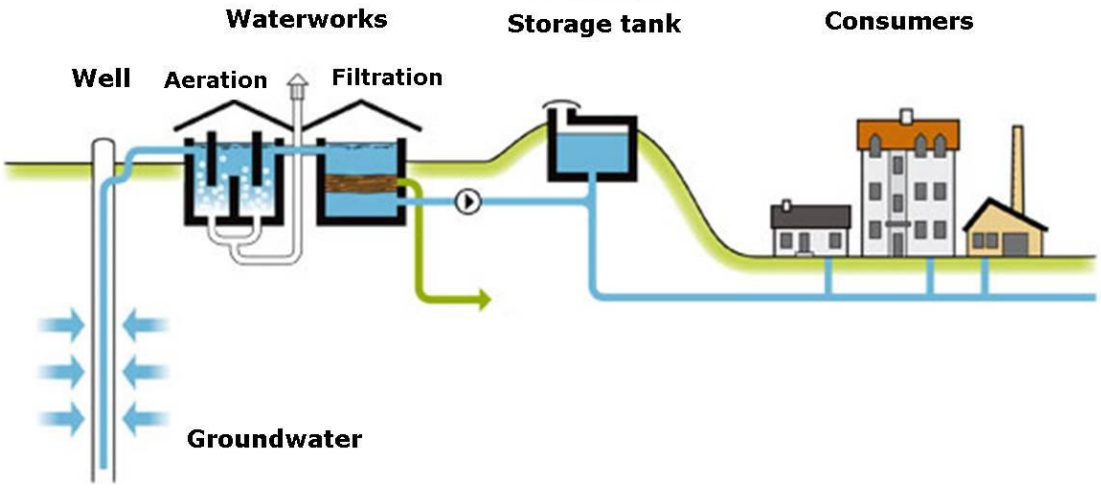


Figure 2. Tradition drinking water treatment in Denmark (VandCenterSyd, 2013).

Recently, microbial communities of biological rapid sand filters have been investigated for their composition, diversity and controlling parameters. The general understanding is that rapid sand filters are dominated by prokaryotes, and that the composition is depended on the groundwater chemistry (Gülay et al. 2016; Palomo et al. 2016; Albers et al. 2015a). Among others, methane oxidizing bacteria were found to be a part of the core taxa of rapid sand filters (Gülay et al., 2016). Filter sand coating is also of importance for microbial colonization and resulted for example in higher microbial densities and ammonium removal rates (Gülay et al., 2014).

2.2.1 Drinking water treatment processes for pesticide removal

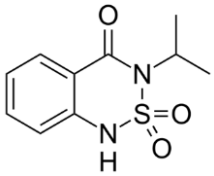
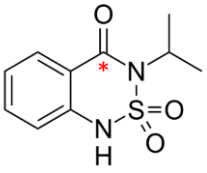
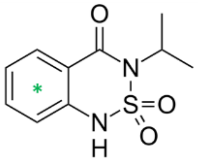
Several advanced water treatment processes, i.e. granular activated carbon (GAC) (Heijman et al., 2002), advanced oxidation (Suty et al., 2004) and membrane biofilm reactors (Modin et al., 2008) can remove trace organic contaminants such as pesticides from the water phase. In drinking water treatment ozonation followed by biological activated carbon (BAC) filtration is frequently used for removal of total organic carbon (TOC), especially micropollutants such as pesticides (Camel & Bermond 1998; Van Der Hoek et al. 1999). However, these processes are often less cost-effective or environmental sustainable than simple treatment processes such as biological rapid sand filtration (Godskesen et al., 2011).

3 Methods

Pesticide removal was investigated at three scales; full-scale waterworks, lab-scale methanotrophic column reactors, and microcosms experiments. Since the main purpose was to investigate the governing mechanisms of pesticide removal in drinking water systems, most investigations were performed in microcosm (for details Hedegaard et al., **I, II, III, IV** and **V**).

Pesticide degradation was measured by different analytical approaches. In general, removal was measured by ^{14}C-pesticide, since this method allowed quantification of removal at very low concentrations (< 1.5 µg/L), and detection of mineralisation. The draw-back of this method is, that it might not be possible to distinguish between the presence of the pesticide or its transformation products. This was especially relevant when investigating the methanotrophic removal of bentazone, where the transformation of bentazone to OH-bentazone was of particular interest and could not be detected by ^{14}C-bentazone (see section 7). Thus, ^{14}C-bentazone was supplemented with measurements of bentazone removal and transformation quantified by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD), Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) and high-resolution mass spectrometry (HRMS) at both high (1-5 mg/L) and low concentrations (10 µg/L). Also the use of both ^{14}C-carbonyl-bentazone (^{14}C-label marked by red asterisk in table 2) and ^{14}C-ring-bentazone (^{14}C-label marked by green asterisk in table 2) allowed investigations on which parts of the molecule were removed from the water phase and mineralized at low concentrations (< 1.5 µg/L). Altogether, these measurements allowed determination of bentazone removal and identification of transformation products during degradation.

Table 2 Structures, concentration and analytical approaches for bentazone and ^{14}C-bentazone. The asterisk shows the position of the ^{14}C-label in ^{14}C-carbonyl- (red) and ^{14}C-ring-bentazone (green). Analytical approaches included High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD), Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS), High-Resolution Mass Spectrometry (HRMS) and Liquid Scintillation Chromatography (LSC).

Structure			
Name	Bentazone	^{14}C-carbonyl-bentazone	^{14}C-ring-bentazone
Conc.	10 µg/L to 5 mg/L	1 µg/L (and fraction of 1 mg/L)	1 µg/L
Analysed by	HPLC-DAD, HRMS And LC/MS/MS	LSC	LSC

4 Pesticide removal in biologically active sand filters

Biological treatment has been suggested as a potentially cost-effective and environmental sustainable alternative for removal of trace contaminants in drinking water (Benner et al., 2013). Therefore, we investigated whether a full-scale waterworks could remove pesticide from contaminated groundwater, and subsequently whether filter sand from different groundwater-based waterworks showed an omnipresent pesticide degradation potential.

4.1 MCPP removal at full-scale waterworks

Full-scale investigations of the phenoxy acid mecoprop (MCPP) was conducted at Kerteminde waterworks, in Denmark. MCPP had been detected below guideline values (0.1 µg/L) in groundwater abstraction wells and in the effluent water from the waterworks for several years. However, from 2002 MCPP was no longer detected in the effluent water, even though it was still detected in the abstraction wells (Figure 3) (Hedegaard et al., **I**).

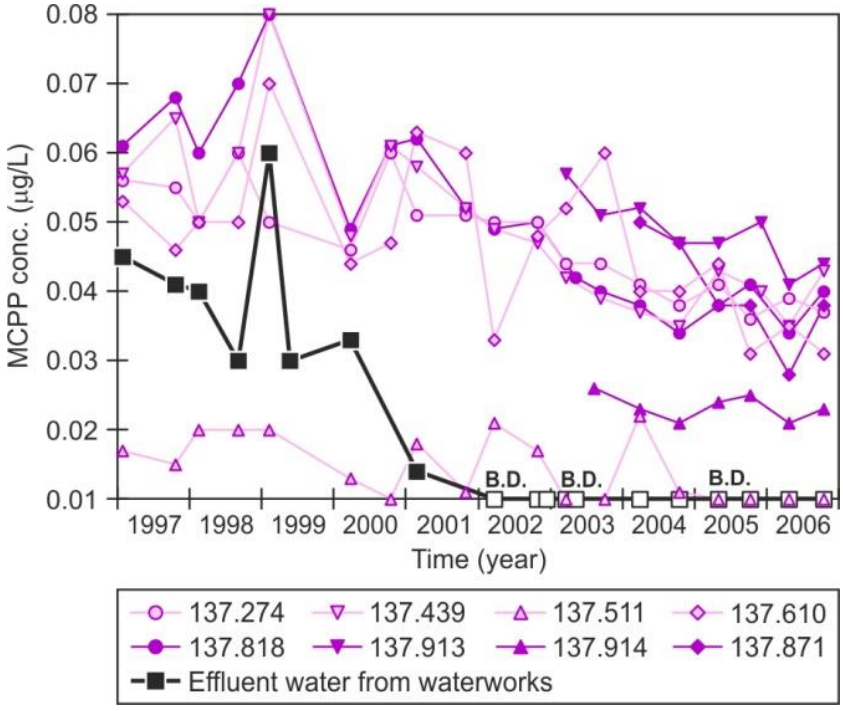


Figure 3. Concentration of the herbicide mecoprop (MCPP) at Kerteminde waterworks. Measured in water abstraction wells (well numbers on figure) and in effluent water from the waterworks (GEUS & Danish Ministry of Energy Utilities and Climate, 2013) (Hedegaard et al., **I**).

The water treatment at Kerteminde waterworks consists of aeration followed by rapid sand filtration in two parallel filter lines with primary and secondary filters. The secondary rapid sand filters removed MCPP in both filter lines. Filter line 1 (contact time: 63 minutes) removed MCPP to below the detection limit (0.010 µg/L), and Filter line 2 reduced MCPP concentration with more than 50% from 0.046 to 0.025 µg/L, within the contact time of 8 minutes (Figure 4). Subsequently, water from the two filter lines was mixed in the clean water tanks, and MCPP concentration was below the detection limit (Hedegaard et al., **I**). Thus, a full-scale biological rapid sand filter at a Danish waterworks was able to remove a pesticide contamination from drinking water after an adaptation period of > four years (Hedegaard et al., **I**).

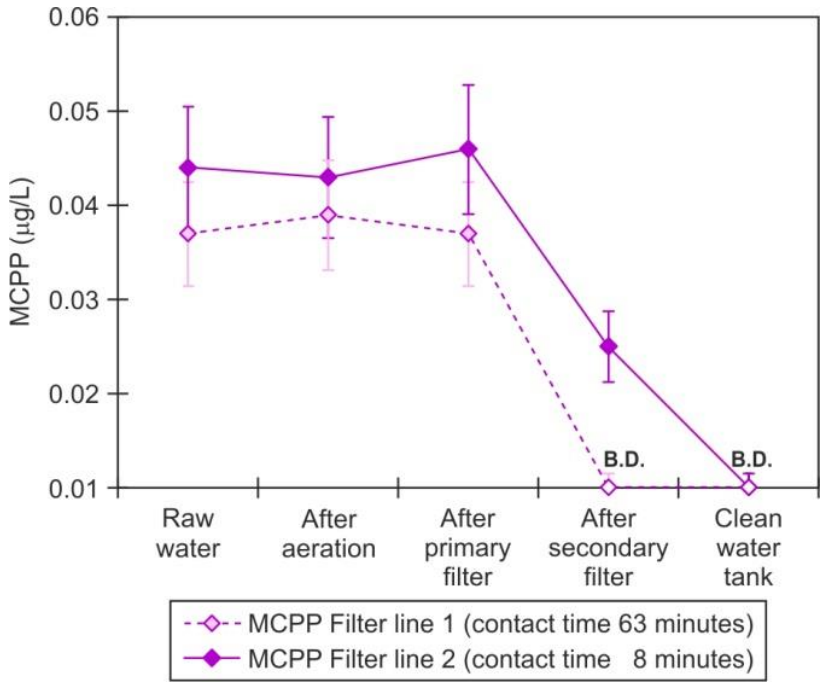


Figure 4. Mecoprop (MCPP) concentration in water phase throughout the treatment process at Kerteminde waterworks. MCPP concentrations in Filter line 1 (contact time 63 minutes) and 2 (contact time 8 minutes) (Hedegaard et al., **I**).

4.2 Removal potential of pesticides in filter sand

Lab-scale studies have shown a biological removal potential of pharmaceuticals in filter sand from biological rapid sand filter treating contaminated groundwater (Zuehlke et al., 2007), and others have found that biological filters used to treat surface water are able to remove pesticides after a six-month adaption period (Zearley and Summers, 2012). To further investigate the pesticide removal potential in filter sand from rapid sand filters used for treatment of groundwater, the degradation of pesticides (MCPP, dichlorprop, glyphosate

and *p*-nitrophenol) at low concentrations (< 1 µg/L) was investigated in filter sand from three different waterworks.

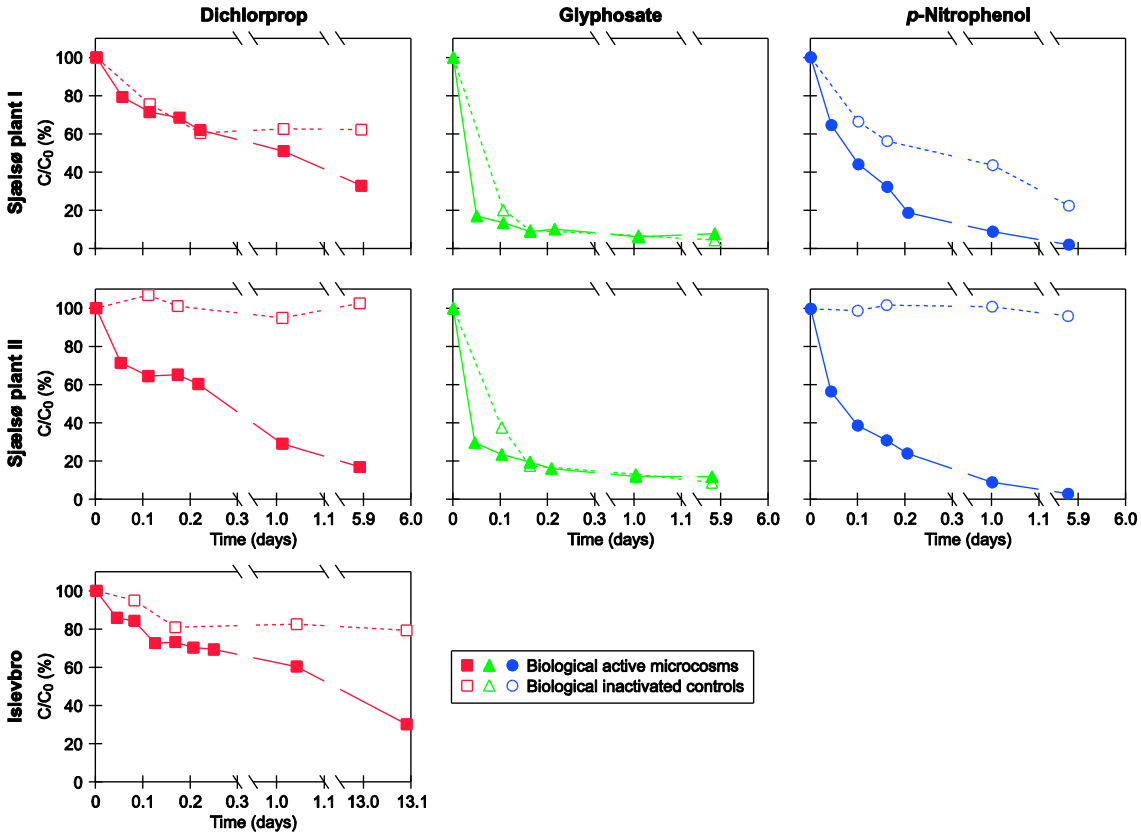


Figure 5. Removal potential of pesticides in filter sand from rapid sand filters. Removal of dichlorprop, glyphosate and *p*-Nitrophenol with filter sand from Sjælsø Plant I and II and Islevbro (modified from Hedegaard & Albrechtsen, 2014)

All three investigated filter sands removed pesticides from the water phase (Figure 5). Removal of MCPP (except at Sjælsø Plant I), dichlorprop and *p*-nitrophenol was partially biological, since more was removed in microcosms than in abiotic controls. The initial removal of glyphosate though, was due to abiotic processes (Figure 5). The largest microbial degradation was observed in the filter sand from Sjælsø Plant II (SPII), which received methane-rich groundwater. Here degradation led to a partial mineralisation of the pesticides (Hedegaard and Albrechtsen, 2014).

The herbicide bentazone was also degraded rapidly in filter sand from SPII (Hedegaard and Albrechtsen, 2014). The measured bentazone removal rate was used to estimate whether removal was adequate to manage a hypothetical contamination at a full-scale waterworks (assuming that the removal rate in a full-scale filter was similar to the detected removal rate in microcosms). The microbial bentazone removal rate at 10 µg/L was 3.6×10^{-3} nmoles/h/g_{sand}

(Hedegaard et al., **III**). Typically, removal capacities are stratified in biological filters, as is observed for ammonium, with the largest capacity in the top layer (Lee et al., 2014; Tatari et al., 2016). Assuming the same stratification of the pesticide removal capacity, a full-scale filter would be able to remove 16 mg bentazone $filter^{-1}$ h^{-1} (assuming homogeneous bentazone removal in the top 30 cm with a filter surface area of 50 m^2 at SPII). Compared to this, a hypothetical contamination of bentazone at 0.1 µg/L (the maximum threshold concentration for pesticides in groundwater) would imply a loading rate of 6.2 mg bentazone/h/filter, since the normal flow to the filters is 62 m^3/h. The measured bentazone removal rate in microcosm experiments indicated that the bentazone removal would be sufficient to expect complete removal at a full-scale filter, when concentrations are close to the guideline value (Hedegaard et al., **III**).

Overall, there was a potential for removing pesticides in filter sand from rapid sand filters at waterworks treating groundwater. Additionally, the largest biological removal was detected at Sjælsø Plant II, which differed from the other waterworks by having high concentrations of methane in the raw water (Hedegaard and Albrechtsen, 2014). At Kerteminde waterworks MCPP was removed after an adaptation period of > four years (Hedegaard et al., **I**). While in filter sand from other waterworks the immediate pesticide removal showed that the degrading organisms were already present in the full-scale filters (Hedegaard and Albrechtsen, 2014).

Summary: ***Section 4 - Pesticide removal in biologically active sand filters***

- A full-scale rapid sand filter at a Danish waterworks removed a MCPP contamination from drinking water after an adaptation period of > four years.
- Microcosm experiments with filter sand from three waterworks showed:
  - A general pesticide removal potential in filter.
  - The largest biological removal was detected at a waterworks characterized by having high concentrations of methane in the raw water.
  - Removal of all the investigated pesticides began immediately, and thus, the degrading organisms were present in the full-scale filters.
  - Calculations, based on bentazone removal rates in microcosms, indicated that degradation was sufficient to expect removal in a full-scale rapid sand filter.

5 Microbial degradation strategies

Microbial degradation of pesticides may occur via processes known as primary metabolism or co-metabolism (e.g. Alexander, 1994; Boopathy, 2000). Animals, plants, and fungi (Eukaryota) typically transform pesticides for detoxification or by fortuitous metabolism of broad-spectrum enzymes, whereas bacteria (Prokaryota) commonly metabolize them for assimilation of carbon, essential nutrients and/or energy (e.g. Fenner et al., 2013). This process is growth-linked and called primary metabolism (e.g. Alexander, 1994; Boopathy, 2000). Dedicated metabolic pathways and enzymes are thought to be acquired by horizontal gene transfer, point mutation, and gene rearrangements (Top et al., 2002). In the metabolic strategy pesticide degradation is stimulated via primary metabolism (Figure 6).

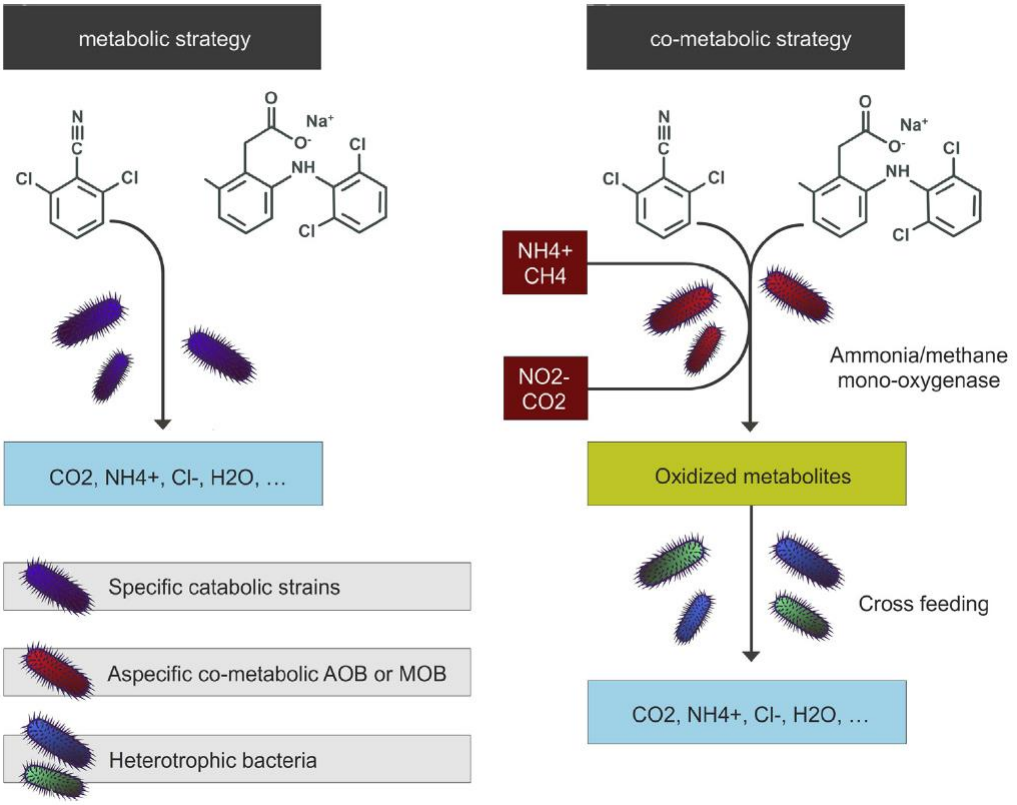


Figure 6. Metabolic and co-metabolic biodegradation strategies. The metabolic strategy is a growth-linked process where microorganisms grow on the trace contaminant and this process leads to mineralisation of the trace contaminant. In the co-metabolic strategy the secondary substrate is oxidized without being used as energy or carbon-source, and this process leads to formation of oxidized transformation products. These transformation products can be utilized as primary substrates for other heterotrophic bacteria (Benner et al., 2013).

Bacterial cells in drinking water treatment systems have developed strategies to uptake and metabolize dozens of different carbon substrates simultaneously, which equips the cell with a kinetic advantage and metabolic flexibility (Egli, 2010).

In the co-metabolic strategy, trace contaminants are degraded along with the primary growth substrate, without being used as an energy, nutrient or carbon source (Dalton and Stirling, 1982). Thus the degrading microorganisms do not gain anything from the secondary substrate (e.g. the pesticide) (Alexander, 1994) and this process leads to a production of oxidized transformation products. These transformation products can subsequently be utilized as primary substrate for other heterotrophs (Figure 6) (Benner et al., 2013).

Depending on the degradation pathways of a specific pesticide, different strategies for stimulation of biological pesticide removal in drinking water treatment can be suggested.

5.1 Metabolic strategy for pesticide removal

Within the metabolic strategy, bioaugmentation with specific pollutant degrader organisms has gained some attention as a method to initiate and/or stimulate pesticide degradation in for example sand filters. In bioaugmentation the pollutant degraders are pre-cultured and added to sand filters (Benner et al., 2013). Thus the pesticide contamination serves as substrate for the added pollutant degraders, which starts growing in the filter sand, while mineralizing the target pesticide (Figure 6). The common groundwater pollutant 2,6-dichlorobenzamide (BAM) can be mineralized in sand filter material, but with low mineralization rates (Vandermaesen et al., 2016). Thus, bioaugmentation of rapid sand filters with a BAM degrading strain, *Aminobacter* sp. MSH1, has been suggested as a promising strategy to remove BAM at waterworks (Albers et al., 2015b). Therefore, rapid sand filters receiving groundwater with low BAM concentrations (0.2 µg/L) were bioaugmented with *Aminobacter* sp. MSH1, and initially BAM was successfully degraded. However, over time (2-3 weeks) the *Aminobacter* sp. MSH1-bacteria was lost from the sand filters mainly during backwash (Figure 7) (Albers et al., 2015b).

For some pesticides, degrader genes are found in indigenous microbial communities, and so target genes for degradation of phenoxy acids; *tfdA, rdpA* and *sdpA* were expressed in an indigenous soil microbial community when exposed to (*RS*)-dichloprop (Paulin et al., 2010). A strategy to stimulate degradation of these pesticides in filter sand could therefore be to enrich a naturally occurring

degrader population. Applying this strategy, MCPP, dichlorprop and 4-CPP degraders were successfully enriched in a biological rapid sand filter during three months operation (Feld et al., 2015). After this period, 15-30% of the MCPP, dichlorprop and 4-CPP was removed in the sand filter (Feld et al., 2015).

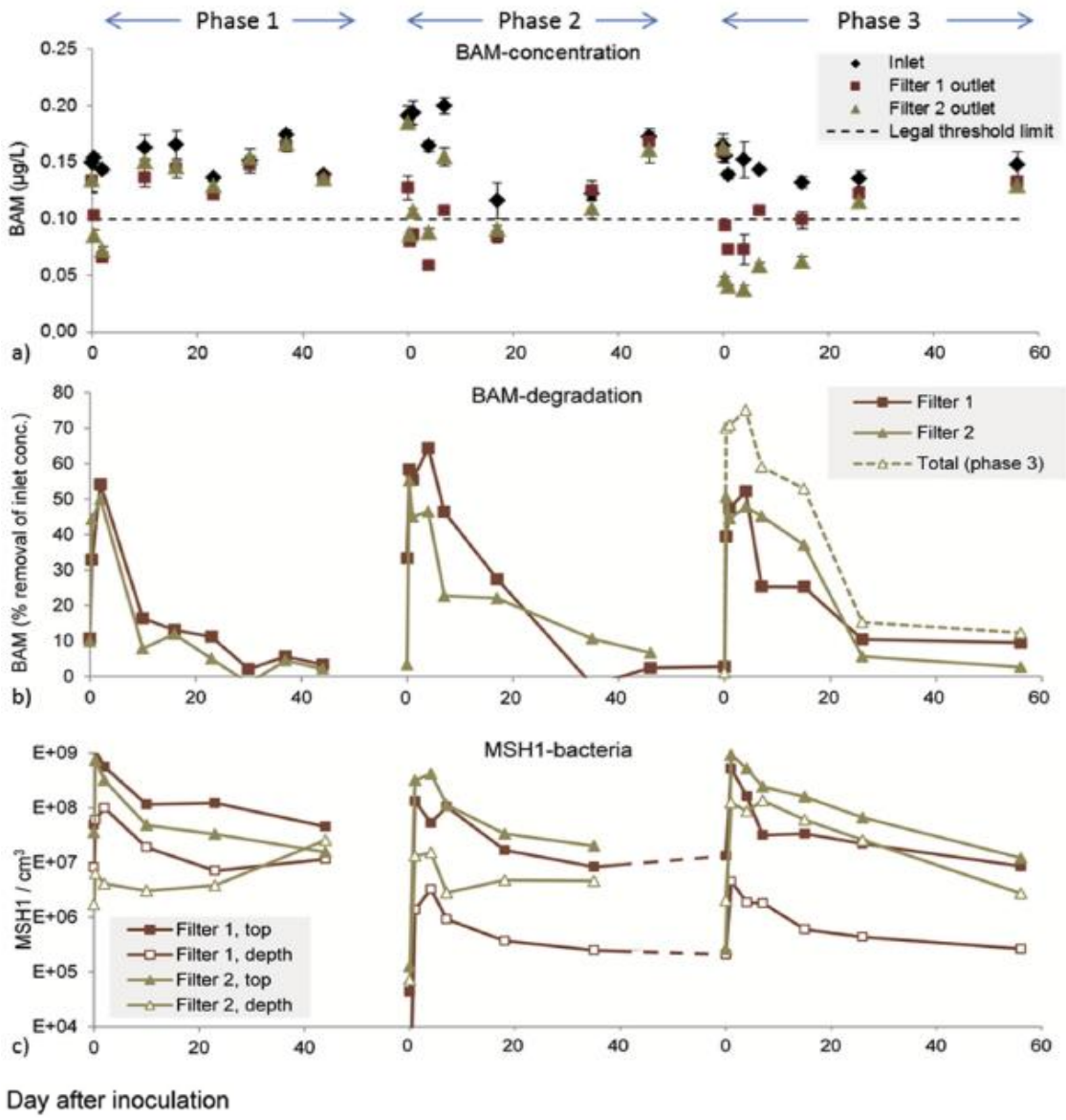


Figure 7. BAM degradation and density of *Aminobacter* sp MSH1 in pilot waterworks sand filters. A) Concentration of BAM in in- and out-let water from the two filters. During phase 3, the filters were run in series, and the BAM concentration shown for the outlet of filter 2 therefore represents the concentration after passage through both filters. Error bars are average deviation fron the mean of three replicate water samples. B) BAM removal efficiency as % of inlet concentration. C) MSH1 density as determined by qPCR analysis of the *bbdA* gene. In all graphs the first data point in each phase is just before inoculation (modified from Albers et al. (2015b)).

These findings show, that there can be a great potential in taking advantage of natural microbial communities and enrich specific degraders in sand filters (Feld et al., 2015). However, they also illustrate the difficulty of sustaining cultivated strains when introduced into a new environment, where concentration levels of their growth substrate, the pesticide, is low and fluctuating (Albers et al., 2015b).

5.2 Co-metabolic pesticide removal

Another promising strategy to stimulate biological removal in biological active sand filters is co-metabolic degradation of the trace contaminants (Benner et al., 2013; Verstraete and De Vrieze, 2017). Co-metabolism has gained a lot of attention, since it allows microbial degradation of trace contaminants at low concentrations, by controlling the presence of the primary substrate, which can be relatively inexpensive and nontoxic (e.g. CH_4, NH_4^+) (Iwamoto and Nasu, 2001; Semprini et al., 1990; Semprini and McCarty, 1991; Semrau et al., 2010).

Examples include ammonium- and manganese oxidizing bacteria, degrading 17α-ethinylestradiol in wastewater treatment (Forrez et al., 2009), and ammonium oxidizing bacteria, degrading different pharmaceuticals in water treatment systems (Dawas-Massalha et al., 2014; Kassotaki et al., 2016; Xu et al., 2017). However, direct evidence for co-metabolic degradation can be difficult to establish. For example, was biotransformation of trace contaminants was not directly associated with ammonia monooxygenase activity although it was linked to ammonia removal (Helbling et al., 2012).

5.2.1 Methanotrophs

Methanotrophs are aerobic gram-negative bacteria, which utilize methane as their carbon and energy source (Hanson and Hanson, 1996). Many methanotrophs are known to produce extracellular polymeric substances (EPS), which can take form as copious slime (Hilger et al., 1999; Hou et al., 1979). An excessive production of EPS can be problematic in water treatment systems, since biofouling can reduce the efficiency of the technical processes (Flemming et al., 1997).

Methane monooxygenase (MMO) is the key enzyme in methane oxidation and can oxidize trace contaminants co-metabolically (Dalton and Stirling, 1982; Semrau et al., 2010). Methanotrophic co-metabolic degradation is especially well studied for trichloroethylene (TCE) and other chlorinated aliphatic hydrocarbons (i.e. Oldenhuis *et al.*, 1989; Alvarez-Cohen and McCarty, 1991;

DiSpirito *et al.*, 1991; Alvarez-Cohen *et al.*, 1992), but the MMO can also degrade pharmaceuticals and chemical additives such as sulfamethoxazole and benzotriazole (Benner et al., 2015). MMO can either be the particulate, membrane bound enzyme (pMMO), which is expressed by nearly all known methanotrophs, or the soluble cytoplasmic enzyme (sMMO), which is only expressed by some methanotrophs. Generally, oxidation by pMMO is limited to alkanes, with up to five carbon atoms, while sMMO is less specific and able to oxidize alkanes, esters, cyclic alkanes and aromatic compounds (Burrows et al., 1984; Semrau et al., 2010). sMMO is expressed at low copper to biomass ratios, whereas pMMO increases with increasing ratio (Semrau et al., 2013; Sirajuddin and Rosenzweig, 2015). The MMO activity can be inhibited by acetylene (Prior and Dalton, 1985; Sullivan and Chase, 1996).

5.2.2 Enrichment of methanotrophs in reactors

To investigate methanotrophic co-metabolic degradation of pesticides, methanotrophic enrichment cultures were cultivated in four replicate continuous flow column reactors, which were filled with expanded clay and initially augmented (2% v/v) with filter material from Sjælsø waterworks (Figure 8).

The abundance of methanotrophs increased from 8.5×10^5 cells/g_{sand} in the rapid sand filter to 2.55×10^7 cells/g biomass and carrier material ($g_{B\&C}$) after one year of enrichment, and thus methanotrophs were successfully enriched in the column reactors. Meanwhile, the fraction of methanotrophs, compared to the total number of bacteria, increased from 1.3% to 12% and was thereby larger in the column reactors, than in the rapid sand filters (Papadopoulou et al., **IV**).

5.2.3 Transformation of pesticides in the column reactors

Removal of 10 pesticides in the methanotrophic column reactors was investigated. The pesticides were divided into three groups based on their removal potential. The first group (1) BAM, Bromoxynil, Chlorotoluron and Ioxynil, were not removed in the column reactors. The pesticides in the second group (2) Diuron, Isoproturon, and Linuron were all partly removed, both in column reactors with methane and in reactors starved for methane (starved for one week). The removal was lower or at the same level though, as removal in the non-inoculated control column (fed with drinking water), and could thus not be ascribed to a methanotrophic activity. The third group (3) included the two phenoxy acids. None of the phenoxy acids were significantly removed in the control or in presence of methane, but in the column reactor with methanotrophs starved for methane, both MCPA ($P=0.0213$, unpaired *t*-test) and MCPP ($P=0.0208$, unpaired *t*-test) were significantly removed (Table 2).

Hence, methane starvation stimulated the phenoxy acids removal (Papadopoulou et al., **IV**).

We calculated a limitation of the technical system, showing that for most pesticides the expected removal in the column reactors was lower than the detectable removal (Papadopoulou et al., **IV**). Methanotrophic co-metabolic pesticide degradation was further investigated with the methanotrophic enrichment cultures in microcosms to increase contact time and methane oxidation.

Figure 8. Reactor set-up for enrichment of methanotrophic biomass. Filter material consisted of Filtralite NC 0.8-1.6, and were originally augmented (2% v/v) with filter material from the prefilters at Sjælsø waterworks. A) Reactors, B) Methanotrophic biomass from reactor in microcosm filled with water, C) Microcosm for experiment and D) Reactor set-up.

Summary: *Section 5 - Microbial degradation strategies*

- Different strategies for stimulation of biological pesticide removal in drinking water treatment has been suggested.
- Metabolic strategy
  - In the metabolic strategy pesticide degradation is stimulated via primary metabolism where the pesticides are metabolized for assimilation of carbon, essential nutrients and/or energy.
  - Bioaugmentation of rapid sand filters with a BAM degrading strain, has had limited success, since the *Aminobacter* sp. MSH1-bacteria were lost from the sand filters (mainly during backwash).
  - Natural occurring MCPP, dichlorprop and 4-CPP degraders were successfully enriched in a biological sand filter for treatment of contaminated groundwater
- Co-metabolic strategy
  - Another strategy is co-metabolic degradation, during which the pesticides are degraded along with a primary growth substrate, without being used as an energy, nutrient or carbon source
  - Methanotrophs co-metabolically degrade trace contaminants by methane monooxygenases (MMO), the key enzyme in methane oxidation.
  - Methanotrophs were grown in column reactors inoculated with filter sand from Sjælsø waterworks.
  - The phenoxy acids, MCPA and MCPP, were significantly removed in column reactor starved for methane, while no removal was detected in the control or in presence of methane.
  - A calculated limitation showed that for most pesticides, the expected removal in the column reactors was lower than the detectable removal

6 Degradation of phenoxy acids

Degradation of phenoxy acids has primarily been focused on primary metabolism. However, in the methanotrophic column reactors, the phenoxy acids (MCPA and MCPP) showed the highest degradation potential (Papadopoulou et al., **IV**). Therefore degradation of phenoxy acids in the methanotrophic enrichment culture was investigated. Subsequently, the association between methane oxidation and phenoxy acid degradation in filter sand from rapid sand filters was investigated.

6.1 Methanotrophic phenoxy acid degradation

Dichlorprop is the most frequently detected phenoxy acid in Danish groundwater (GEUS & Danish Ministry of Energy Utilities and Climate, 2016a). The methanotrophic dichlorprop degradation potential was therefore investigated in microcosm experiments (Figure 9).

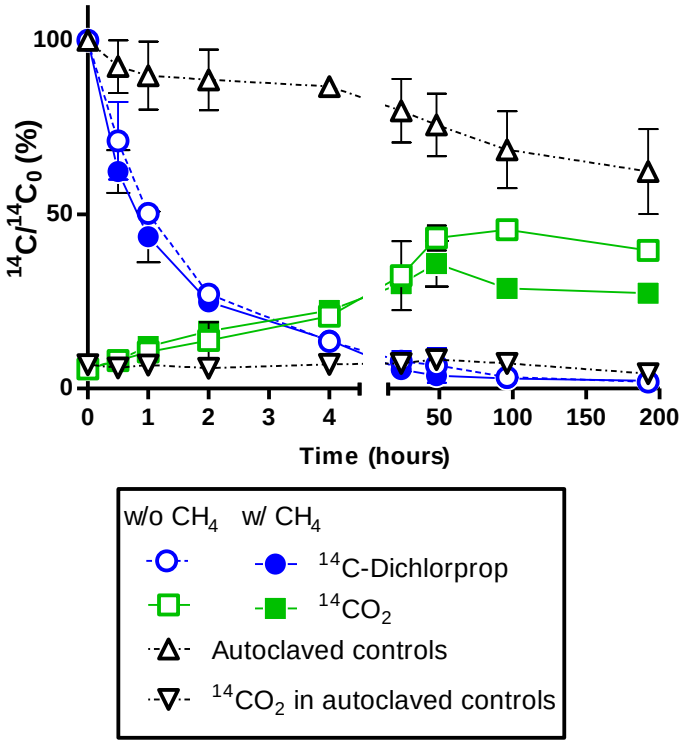


Figure 9. Dichlorprop removal and mineralization in methanotrophic enrichment culture. Microcosms with 10 g enrichment culture and carrier material, 100 mL tap water and ^{14}C-dichlorprop (initial concentration 0.9 µg/L) with (w/ CH_4) (1.5 mg/L) and without methane (w/o CH_4). Mean values and standard deviation of the dichlorprop concentration in the water ($^{14}C/^{14}C_0$) and the $^{14}CO_2$ production ($^{14}CO_2/^{14}C_0$) from mineralization of dichlorprop (all in triplicates) (Papadopoulou et al., IV).

The methanotrophic enrichment culture removed dichlorprop rapidly. The presence of methane did generally not affect dichloprop removal, but dichlorprop was mineralized to a larger extent in absence versus presence of methane (Figure 9) (Papadopoulou et al., **IV**). Because the presence of methane affected dichlorprop mineralization, but not removal, it was suggested that non-methanotrophs were responsible for the initial dichlorprop degradation step, while hydroxylation of the benzene ring was associated with methane oxidation (Papadopoulou et al., **IV**). Other studies proposed that dioxygenases were responsible for the initial cleavage of the MCPP, while monooxygenases were responsible for subsequent ring-hydroxylation (Tett et al., 1997).

6.2 Degradation of phenoxy acids in filter sand

The MCPP removal in filter sand from 10 different waterworks, with varying concentrations and absence of methane in the raw water (Hedegaard et al., **V**), was investigated to identify the determining parameters for removal. All the investigated filter sands removed MCPP biologically (Figure 10) (Lee et al., 2017).

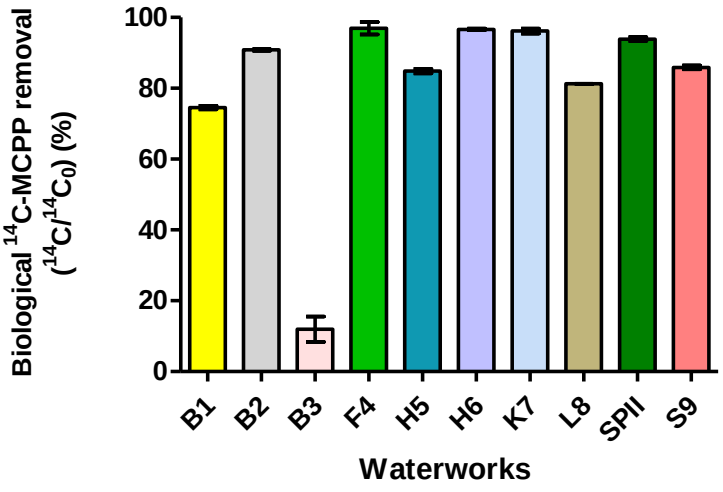


Figure 10. Biological removal of mecoprop (MCPP) at 10 waterworks treating groundwater with different concentrations and absence of methane (SPII=Sjælsø Plant II) (modified from Lee et al., 2017).

Neither MCPP removal nor mineralization correlated with the concentration of methane in the raw water of the rapid sand filters (Table 3), and so MCPP removal was not associated with methanotrophic activity in the rapid sand filters. Unexpectedly, MCPP mineralisation showed a significant correlation with the maximum iron concentration in the raw water (P=0.0396). To the author's knowledge, iron-oxidizing bacteria are not known to perform co-metabolic

degradation of trace contaminants. However, the iron concentration determines the amount of iron oxide precipitation on the filter sand, and thereby it affects the coating of the filter sand (e.g. Dimitrakos Michalakos et al., 1997). Filter sand coating can support microbial colonization and result in higher microbial densities and ammonium removal rates (Gülay et al., 2014). Thus, the correlation between MCPP mineralization and iron concentration could be due to a general increase of microbial colonization at higher iron concentrations. Further studies are needed to confirm this.

Table 3. Correlation between water chemistry and MCPP removal and mineralization in filter sand from 10 different rapid sand filters. Pearson correlations and P-values (two-tailed) between the maximum concentration of the different water quality parameters at the waterworks and the biological MCPP removal and mineralization (data from Lee et al., 2017; Hedegaard et al., **V**).

Maximum concentration of:			**Ammonium /ammonia**	**Iron**	**Manganese**	**Methane**
MCPP	Removal	Pearson r	-0.06	0.36	-0.30	0.17
		P-value	0.8771	0.3081	0.3975	0.6396
MCPP	Mineralization	Pearson r	-0.14	**0.66**	-0.39	0.40
		P-value	0.6049	**0.0396**	0.2705	0.2548

Other investigations showed that natural occurring phenoxy acid degraders were enriched over three month in sand filters treating contaminated groundwater (Figure 11) (Feld et al., 2015). The adaptation period of > four years for initiation of MCPP removal at Kerteminde waterworks also indicated that a specific degrader population had to establish in the filters before removal initiated. The omnipresent biological degradation potential of phenoxy acids in filter sand, no matter the water chemistry, was thus most likely due to primary metabolism, and the target genes might be wide spread in rapid sand filters. Exposing rapid sand filters to phenoxy acid contaminated water can induce an enrichment of naturally occurring specific degraders, leading to enhanced degradation over time (Feld et al., 2015), as was observed at Kerteminde waterworks (Hedegaard et al., **V**). Based our studies and literature it is therefore suggested that phenoxy acid degradation can be stimulated by enrichment of naturally occurring specific degraders by exposing sand filters to phenoxy acid contaminations. Further studies should investigate the general presence of phenoxy acids degraders in filter sand, and whether bioaugmentation or enrichment of naturally occurring specific degraders could be viable strategies to initiate or stimulate phenoxy acid degradation at waterworks.

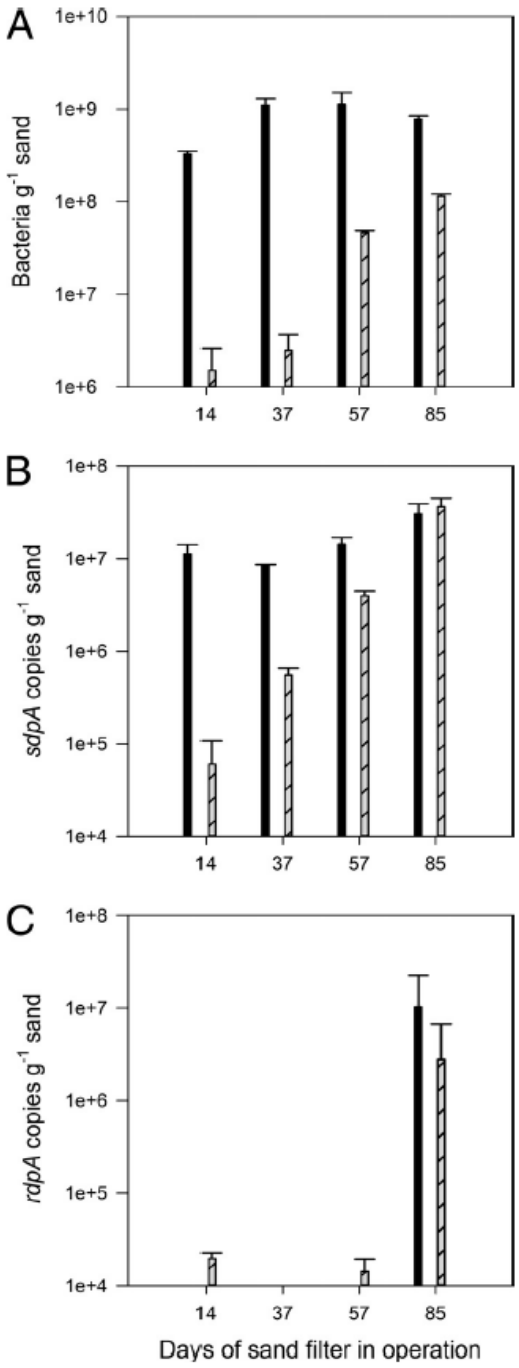


Figure 11. Development of total bacteria and phenoxy acid degradation genes in sand filter, during treatment of MCPP, DCPP and 4-CPP contaminated groundwater. Development of the total number of bacteria (A), number of sdpA gene copies (B), and number of rdpA copies (C) in the top (black bars) and depth (hatched bars) of the sand filter. The numbers were determined by qPCR of the 16S rRNA, sdpA, and rdpA genes. The number of bacteria was estimated by assuming an average of 4.5 16S rRNA gene copies per cell. Due to the presence of PCR-inhibitory substances in the template DNA, the detection level for sdpA and rdpA was higher for the top filter samples (10^5 copies per g sand) than the depth samples (10^4 copies per g sand). The error bars show the standard deviation for triplicate DNA extractions (Feld et al., 2015).

Summary: *Section 6 - Degradation of phenoxy acids*

- Methanotrophic contribution to phenoxy acid degradation
  - In the methanotrophic enrichment culture, dichlorprop mineralization increased in the absence of methane, while dichlorprop removal was unaffected by the presence of methane.
  - It was suggested, that non-methanotrophs were responsible for the initial dichlorprop degradation step, while hydroxylation of the benzene ring was associated with the methane oxidation.
- Phenoxy acid degradation in filter sand
  - MCPP removal and mineralization in filter sand from 10 waterworks did not correlate with the methane concentration in the raw water.
  - MCPP mineralization correlated with the iron concentration in the raw water of the waterworks, which could be due to a general increase of microbial colonization at higher iron concentration.
  - The omnipresent biological degradation potential of phenoxy acids in filter sand was most likely due to presence of specific degraders.
- Stimulation of phenoxy acid degradation in filter sand
  - Further studies should investigate whether bioaugmentation or enrichment of naturally occurring specific degraders in rapid sand filters could be viable strategies to initiate or stimulate phenoxy acid degradation at waterworks.

7 Methanotrophic degradation of bentazone

Bentazone removal was observed in filter sand from rapid sand filters receiving raw water with high methane concentrations (Sjælsø Plant II) (Hedegaard and Albrechtsen, 2014). Ring-hydroxylation is a common initial step in bentazone transformation (Huber and Otto, 1994), and since MMO oxidizes aromatic rings co-metabolically (Semrau et al., 2010), we speculated that methanotrophs were essential for the initiation of bentazone degradation in filter sand (Figure 12). The methanotrophic enrichment culture was used to investigate the interaction between methane oxidation and bentazone removal. The position of the ^{14}C-lables in ^{14}C-carbonyl-bentazone (red asterisk) and ^{14}C-ring-bentazone (green asterisk), indicated that ring-hydroxylation of bentazone by MMO, could not be detected by ^{14}C-bentazone, as long as OH-bentazone is still present in the water phase. Therefore, several analytical approaches (^{14}C-bentazone, HPLC-DAD, LC/MS/MS and HRMS) were applied to quantify bentazone transformation. The purpose of investigating bentazone degradation in the methanotrophic enrichment culture from rapid sand filters, was to isolate the initial degradation step in the bentazone transformation from the complex system in the filter sand, and investigate if this step could be connected with the methane oxidation (Figure 12).

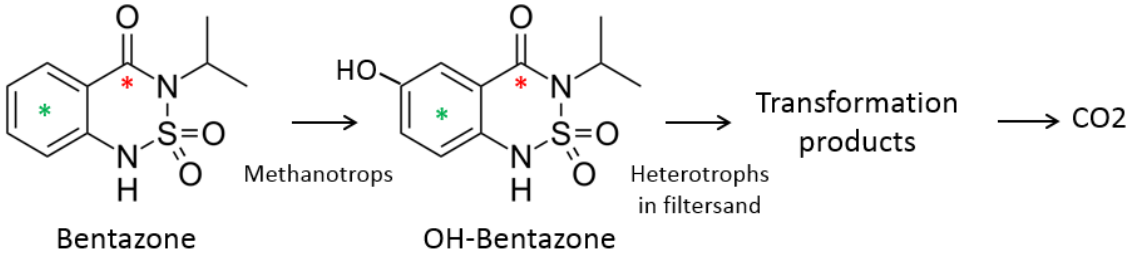


Figure 12. Hypothesized contribution of methanotrophs to the degradation of bentazone. Asterisks marks the position of ^{14}C-labels in bentazone.

7.1 The effect of methane on bentazone removal

The methanotrophic enrichment demonstrated a lower bentazone removal rate in microcosms without methane compared to microcosms with methane (Figure 13) (Hedegaard et al., **II**). Four bentazone transformation products (6-OH-bentazone, 8-OH-bentazone, isopropyl-OH-bentazone and dihydroxy-bentazone) accumulated in the water phase during bentazone degradation by the methanotrophic enrichment culture (Figure 13) (Hedegaard et al., **II**). Four

times more isopropyl-OH-, 132 times more 6-OH- and 85 times more 8-OH-bentazone were observed in the presence of methane compared to cultures without methane (21 days). Additionally, di-OH-bentazone formation was only observed in the presence of methane (Figure 13). Thus, the presence of methane stimulated the bentazone removal and the formation of hydroxy-transformation products by the methanotrophic enrichment culture (Hedegaard et al., **II**).

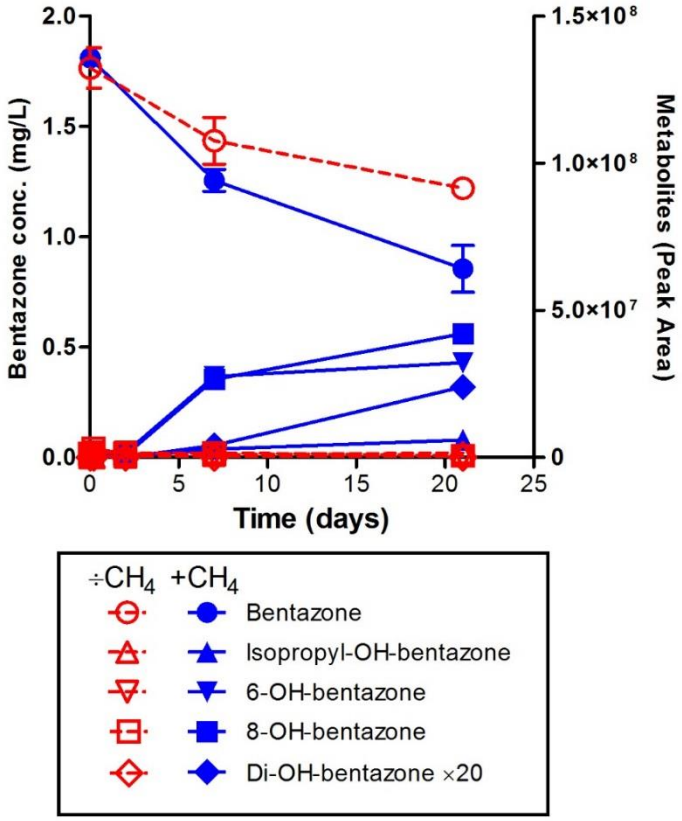


Figure 13. Effect of methane on removal of bentazone and formation of bentazone transformation products by the methanotrophic enrichment culture. Bentazone measured by HPLC-DAD (duplicates) and isopropyl-OH-, 6-OH-, 8-OH-bentazone and di-OH-bentazone measured by High-res-MS as peak areas (single microcosm). Microcosms with 10 g methane enriched biomass and carrier material and 100 mL tap water with methane (approx. 5 mg/L in the water) or without methane (Hedegaard et al., **II**).

7.2 Inhibition of methane oxidation and bentazone removal

To investigate the association between the bentazone removal and methane oxidation, acetylene was added to inhibit methane oxidation. Before acetylene and bentazone were added, all microcosms consumed methane at similar rates of 1.3-2.0 µmole methane/h/$g_{b\&c}$ (Figure 14B, time period: -5 to -1 days). The

methane consumption in the inhibited microcosms stopped when adding acetylene (-1 day) (Figure 14B) (Hedegaard et al., **II**). Addition of acetylene also halted bentazone removal (Figure 14A) (Hedegaard et al., **II**).

After bentazone addition, the methane consumption was 1.5 µmole CH4/h/$g_{b\&c}$ at low (1 µg/L) bentazone concentrations, which was similar to before bentazone addition (Figure 14B). In contrast, at high bentazone concentration (1 mg/L), the methane consumption decreased to 0.6 µmoleCH4/h/$g_{b\&c}$ (Figure 14B). Thus, a high bentazone concentration led to a significantly lower ($P<0.0001$) methane consumption rate, than at low bentazone concentration, which indicated one-way competitive inhibition of bentazone towards methane oxidation (Hedegaard et al., **II**).

Overall, a suite of evidence supported that bentazone was co-metabolically transformed to hydroxy-bentazone by a methanotrophic culture enriched from a rapid sand filter (Hedegaard et al., **II**).

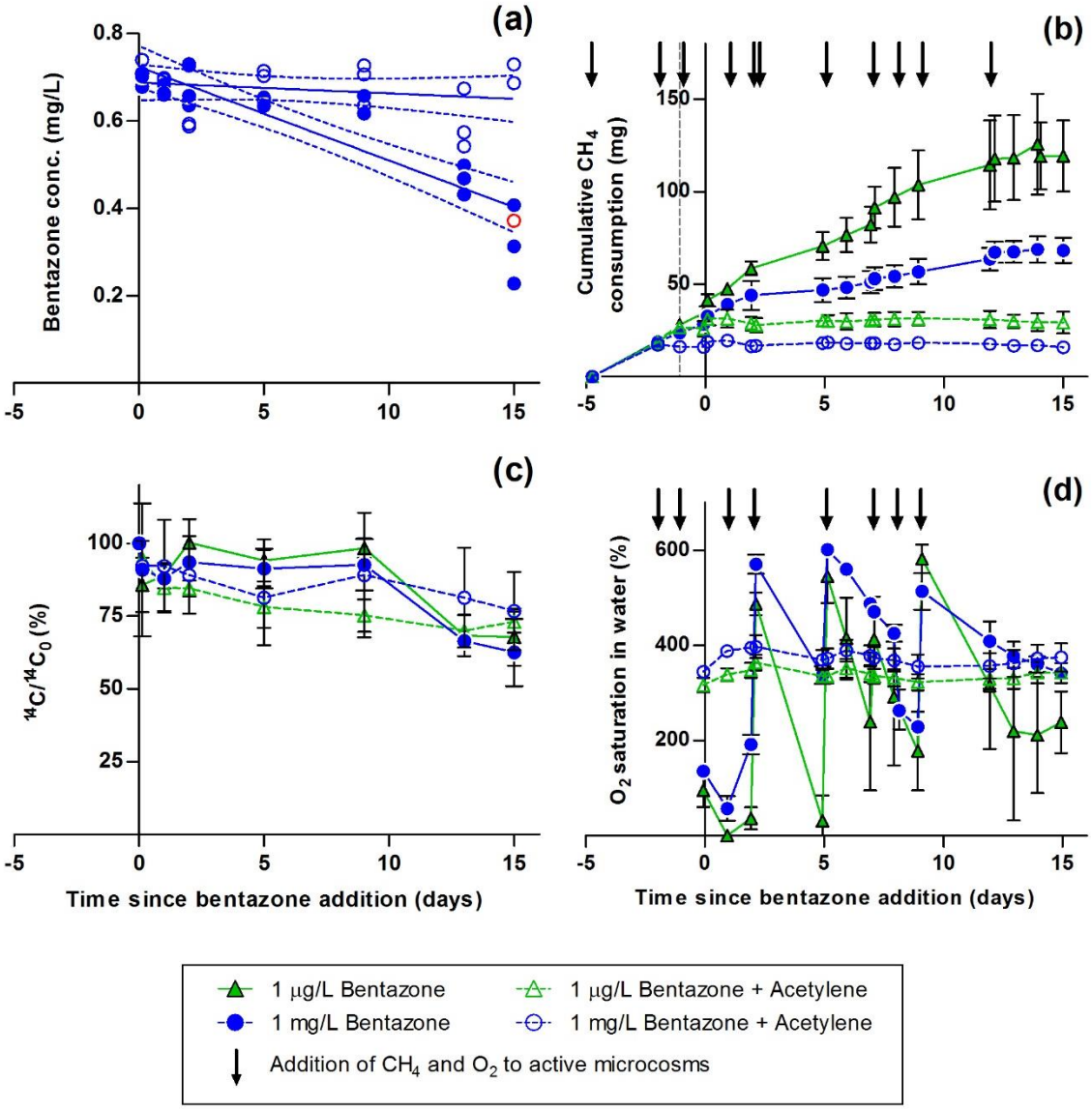


Figure 14 Bentazone removal, methane and oxygen consumption by the methanotrophic enrichment culture. Removal in active microcosms and microcosms with acetylene (both in triplicates) with 10 g biomass and carrier material, 100 mL tap water, approx. 5 mg/L methane and bentazone at high (1 mg/L) and low (1 µg/L) concentrations. A) Bentazone concentration measured by HPLC-DAD, linear regression curves (full lines) with 95% confidence intervals (dotted lines). The red dot marks an outlier (not included in the regression). B) Methane consumption. C) ^{14}C-bentazone concentration given as percentage of initial concentration. D) % oxygen saturation in the water phase. Acetylene (26 mg/L) was added to inhibited microcosms (grey dotted line at time -1 day) prior to bentazone addition (time 0). When $C_{w,CH4}$ < 4 mg/L methane was either added directly or microcosms were flushed with air, and methane and oxygen were added subsequently (2:1 v_{O2}/v_{CH4}) (Hedegaard et al., **II**).

Summary: ***Section 7 - Methanotrophic degradation of bentazone***

- The methanotrophic enrichment culture degraded bentazone, and the presence of methane stimulated the bentazone removal rate.
- Presence of methane also stimulated formation of hydroxylated bentazone transformation products.
- Inhibiting the methane oxidation by acetylene, halted bentazone removal, while high concentrations of bentazone competitively inhibited the methane consumption.
- Thus, a suite of evidence showed that bentazone was co-metabolically transformed to hydroxy-bentazone by the methanotrophic enrichment culture.

8 Methonotrophic bentazone degradation in water treatment

In the methanotrophic culture enriched from the rapid sand filter at Sjælsø waterworks, the initial degradation step of bentazone could be directly connected with the methane oxidation. In this section bentazone removal was investigated in filter sand and other real environmental systems, including both methanotrophs, but also other microorganisms (Figure 12). To this end, the following was investigated;

- The association between the presence/absence of methane and bentazone in active waterworks wells
- The association between methane concentration in the raw water and the biological removal rate of bentazone in the filter sand
- Inhibition of monooxygenases in filter sand and the effect on bentazone removal
- Degradation pathways of bentazone in filter sand
- Bentazone degradation in methanotrophic biomass from aeration tanks

8.1 The presence of methane and bentazone in active waterworks wells

During rain events, seepage and leaching processes allow pesticides to infiltrate into the groundwater (e.g. Malaguerra et al., 2013). However, when aerobic rainwater travels towards methane-rich groundwater, methane and oxygen counter gradients creates growth conditions for methanotrophs (Amaral and Knowles, 1995; Kotelnikova, 2002). We imagined that co-metabolic degradation of bentazone by methanotrophs in real environmental systems could protect methane-rich groundwater against bentazone contaminations. Therefore, we investigated whether bentazone was detected less frequently in methane-rich groundwater compared to groundwater without/low concentrations of methane (< 1 mg/L). Information on the bentazone and methane concentration in active waterworks wells in Denmark was gathered from the Danish '*Jupiter database*' (GEUS & Danish Ministry of Energy Utilities and Climate, 2016b) (Hedegaard et al., **V**). Most wells did neither contain methane (> 1 mg/L) (number of wells: 4822) nor bentazone (number of wells: 610). Bentazone was detected in 113 wells without methane, while it could only be detected in 3 wells with methane (GEUS & Danish Ministry of Energy Utilities and Climate,

2016b) (Hedegaard et al., **V**). A two-sided chi-square test demonstrated a significant correlation between the two parameters (p=0.0053), and so, bentazone was detected significantly less frequently in wells with methane (> 1 mg/L). This result indicated that methanotrophs in real environmental systems protect methane-rich groundwater against bentazone contaminations (Hedegaard et al., **V**).

8.2 Bentazone removal in filter sand

Rapid sand filters represents a complex environment with several abiotic and biological processes occurring simultaneously. In addition to methanotrophs, other groups of bacteria in these filters have co-metabolic properties, for example have both ammonium and manganese oxidizing bacteria been associated with degradation of trace contaminants (Forrez et al., 2009). Therefore, the bentazone removal in filter sand (^{14}C-bentazone at ≤1 µg/L) was investigated for its correlation with governing water quality parameters, more specifically the concentration of ammonium (and ammonia), iron, manganese and methane. Bentazone removal and mineralisation was investigated in filter sand from 14 waterworks with varying concentrations of the different water quality parameters (Lee et al., 2017; Sykyta and Milanovic, 2017; Hedegaard et al., **V**). Bentazone removal did not correlate with the concentration of ammonium, iron or manganese in the raw water, but correlated significantly with the methane concentration (P=0.0016) (Table 4) (Hedegaard et al., **V**).

Table 4 Correlation between water chemistry and bentazone removal at waterworks. Pearson correlations and P-values of the maximum concentration of different water quality parameters at the 14 waterworks (Hedegaard et al., **V**) and the biological removal of bentazone.

Maximum concentration of:			**Ammonium /ammonia**	**Iron**	**Manganese**	**Methane**
Bentazone	Removal	Pearson r	0.07	-0.02	-0.37	**0.76**
		P-value	0.8146	0.9456	0.1879	**0.0016**

Bentazone removal was < 8% (within 5-7 days) for the 10 waterworks with lowest methane concentrations (<2 mg methane/L) in the raw water – and the removal did not induce mineralization in any of these waterworks. Bentazone removal was slightly higher (10%) at Gilleleje waterworks (11 mg methane/L), while filter sand from the two waterworks with highest concentration of methane in the raw water (17-21 mg/L) removed bentazone most efficiently (37-44%) (Figure 15) (Hedegaard et al., **V**).

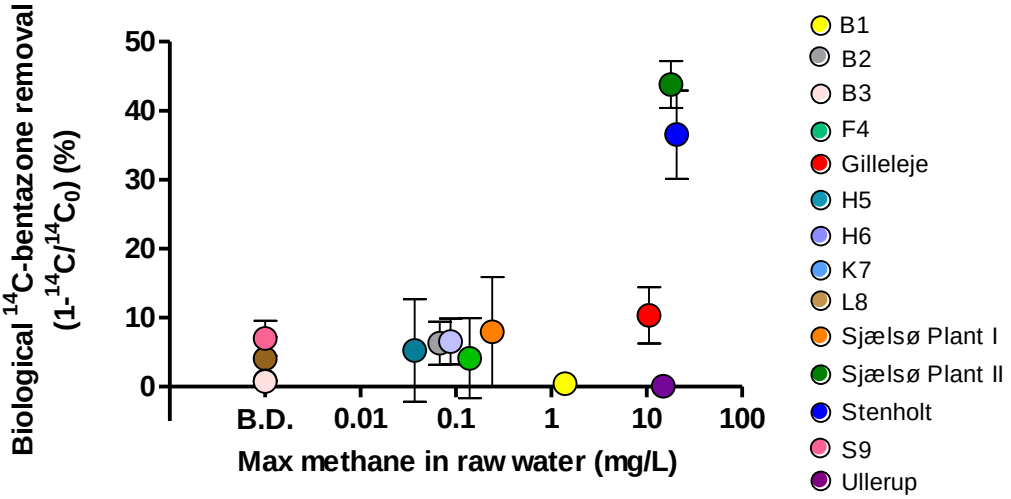


Figure 15 Biological removal of ^{14}C-bentazone (1 µg/L) in 14 different waterworks. Removal was measured in active microcosms (duplicates or triplicates) and autoclaved controls (single microcosm) after 5-7 days. The biological removal is the removal in the active microcosms subtracted the removal in the control (Hedegaard et al., **V**).

Unexpectedly, no biological bentazone removal could be detected in the filter sand from Ullerup waterworks (Figure 15), even though the raw water had high concentrations of methane. However, at this waterworks the copper concentration was higher (1.92 µg/L) in the inlet water, than at the other waterworks (<0.2 µg/L) (Hedegaard et al., **V**). Copper is known to regulate the expression of pMMO versus sMMO, and so, sMMO is only expressed at low copper to biomass ratios (Semrau et al., 2013; Sirajuddin and Rosenzweig, 2015). Recently, addition of copper has been shown to stimulate the ammonium removal capacity in rapid sand filters substantially (Wagner et al., 2016), and the AMO is very similar to the pMMO (Lontoh et al., 2000; Sayavedra-Soto et al., 2011). Thus, the high copper concentrations could indicate that pMMO, rather than sMMO, was expressed at Ullerup waterworks. pMMO is limited to degrade alkanes up to five carbon-atoms, while the sMMO is capable of oxidizing aromatic compounds, such as bentazone (Burrows et al., 1984; Semrau et al., 2010; Trotsenko and Murrell, 2008). Hence, the high copper concentration at Ullerup waterworks could have inhibited the expression of sMMO, and thereby also the degradation of bentazone (Hedegaard et al., **V**). To confirm this, further studies of the microbial community in the different sand filters are needed.

Methane addition positively affected bentazone removal by the methanotrophic enrichment culture. Therefore methane was added to stimulate bentazone removal in filter sand, in several independent experiments. Unexpectedly, the bentazone removal in the filter sand was not significantly affected by the presence of methane (Hedegaard et al., **V**).

8.3 Inhibition of monooxygenases and the effect on bentazone removal

Filter sand from the two waterworks with the largest bentazone degradation (Sjælsø Plant II and Stenholt) was used to investigate the association between bentazone removal and the activity of monooxygenases. Therefore, acetylene, which inhibits both ammonium monooxygenases (AMO) and methane monooxygenases (MMO) (Bédard and Knowles, 1989; Lontoh et al., 2000), was added to microcosms with filter sand.

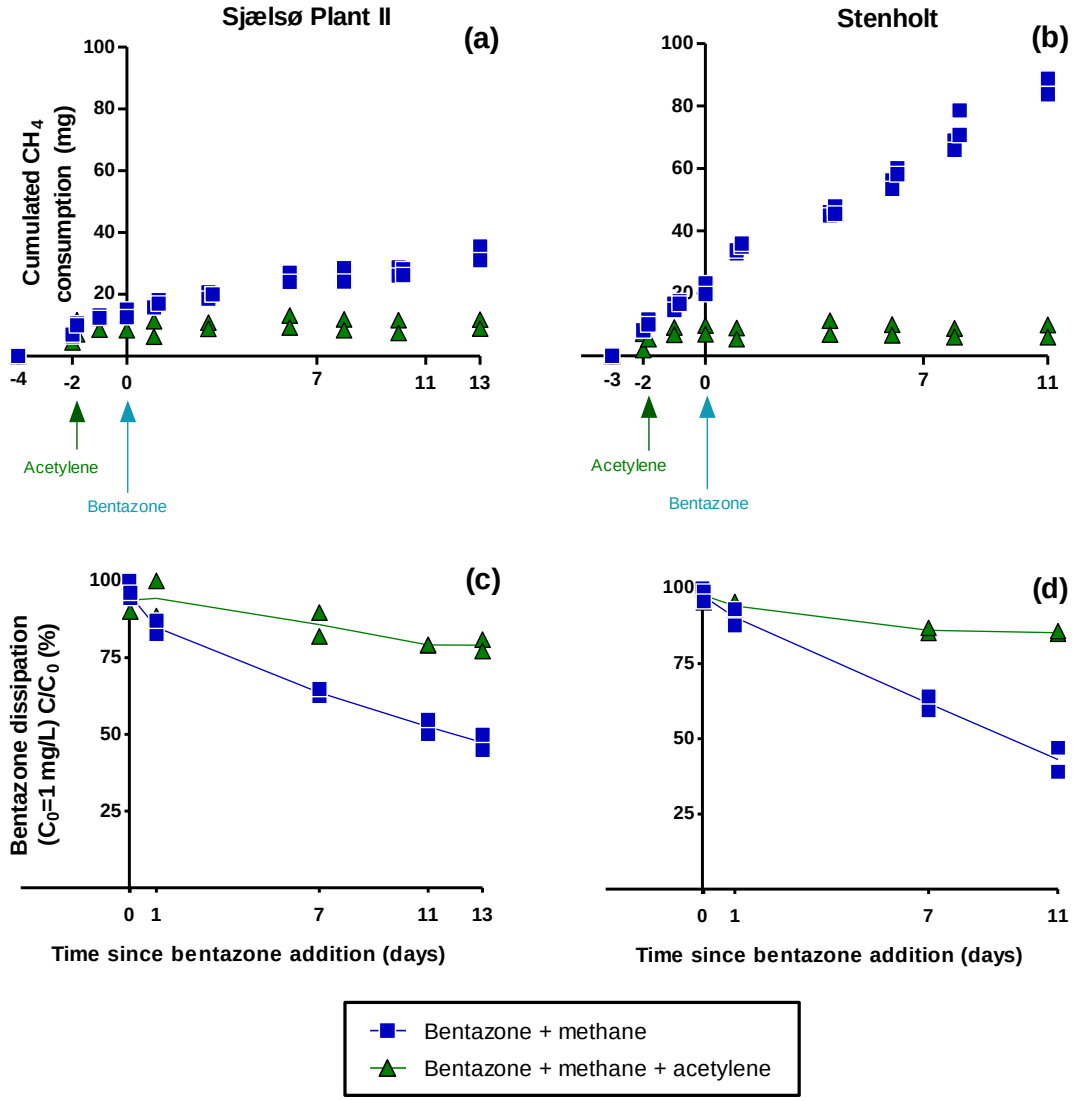


Figure 16 Methane oxidation and bentazone removal and the effect of acetylene. Methane oxidation and bentazone removal (1 mg/L) in filter sand from Sjælsø Plant II (SPII) and Stenholt in active microcosms and microcosms with acetylene (26 mg/L) (in duplicates). A) SPII methane consumption. B) Stenholt methane consumption. C) SPII bentazone removal. D) Stenholt bentazone removal (Sykyta and Milanovic, 2017; Hedegaard et al., **V**).

Filter sand from both waterworks oxidized methane. The methane-consumption stopped immediately after addition of acetylene, and in these microcosms bentazone removal was significantly lower, than in microcosms without acetylene (Sjælsø Plant II: P=0.00043; Stenholt: P<0.0001) (Figure 16) (Hedegaard et al., **V**). Thus, the largest fraction of the bentazone degradation was ascribed to activity of monooxygenases. However, from these experiments, it was not possible to differentiate between the activity of AMO and MMO. A minor bentazone removal was observed in the presence of acetylene, and so a metabolic pathway, which was not associated with monooxygenases also contributed to the removal (Hedegaard et al., **V**).

8.4 Bentazone degradation pathways in filter sand

To understand the bentazone biodegradation process in filter sand, the degradation pathways were identified. Degradation of bentazone in filter sand from Sjælsø Plant II revealed the formation of 10 bentazone transformation products (TPs), which have not previously been identified or ascribed biodegradation (Hedegaard et al., **III**), though some of the TPs have been observed during photocatalytic degradation (Berberidou et al., 2017). The identified bentazone TPs in filter sand clearly indicated the importance of hydroxylation reactions. However, none of the OH-bentazone TPs that were detected in the methanotrophic enrichment culture were detected in filter sand. Degradation of 6-OH- and 8-OH-bentazone, showed that these were very transient in filter sand, and their degradation lead to formation of some of the same TPs as were identified during bentazone degradation. OH-bentazone TPs were thus essential intermediates during bentazone degradation in filter sand (Hedegaard et al., **III**).

Three main biotransformation pathways were identified for bentazone in rapid sand filter (Figure 17); 1) oxidation of the isopropyl-moiety via oxidation of a CH_3 group to the corresponding carboxylic acid, 2) oxidation of the aromatic ring leading to ring cleavage and subsequent decarboxylation reactions, and 3) *N*-methylation followed by oxidation to a carboxylic acid (Hedegaard et al., **III**). Oxidation of the isopropyl-moiety (pathway 1), has only been reported for methanotrophs (Hedegaard et al., **II**), while oxidation of the aromatic ring (pathway 2), is an omnipresent reaction (Hedegaard et al., **II**; Huber and Otto, 1994; Kanungo et al., 2012; Knauber et al., 2000). However, in contrast to bentazone degradation in the methanotrophic enrichment culture, other heterotrophs in filter sand (Figure 6), led to further degradation of bentazone through *both* pathway 1 and 2.

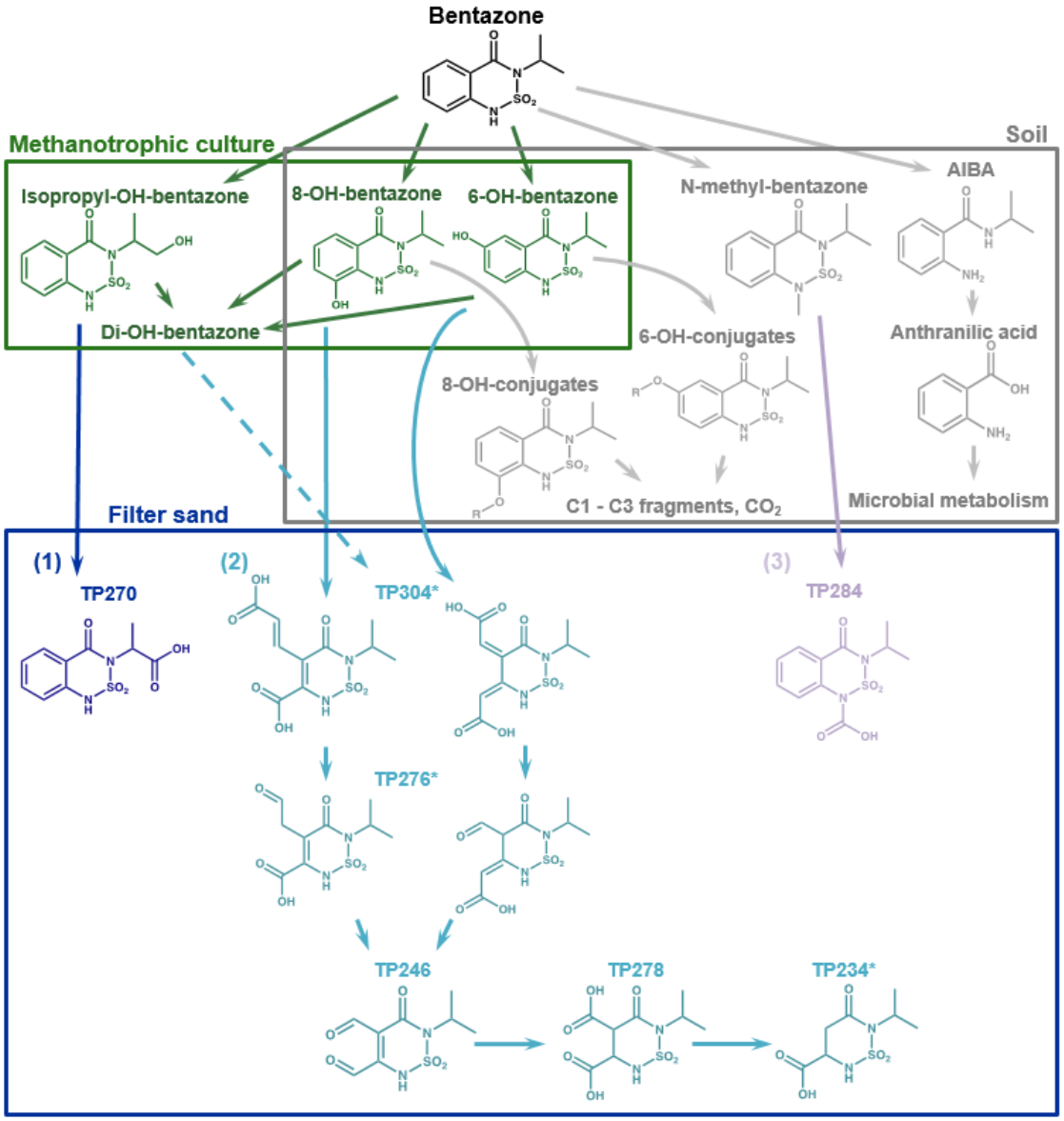


Figure 17. Postulated degradation pathway of bentazone in rapid sand filter. Bentazone degradation pathways in soil (grey)(modified from Huber and Otto, 1994), in contact with methanotrophic culture enriched from a rapid sand filter at Sjælsø waterworks (green)(Hedegaard et al., **II**) and in contact with filter sand from Sjælsø Plant II (blue). Three biodegradation pathways were identified in filter sand, different colors show different pathways (also marked by numbers). * Mark transformation products with several structural isomers. All the suggested structures are tentative (Hedegaard et al., **III)**.

The *N*-methylation (pathway 3) was not observed in the methanotrophic culture. The presence of this pathway in filter sand therefore suggested, that other metabolic pathways assisted the degradation of bentazone in filter sand, and that this degradation was not associated with methanotrophs, but similar to degradation in soils (European Food Safety Authority, 2015). Hence, the diverse microbial community in filter sand (Albers et al., 2015a; Gülay et al., 2016; Palomo et al., 2016) represented an environment, where several different

microbial processes interacted and contributed to a rapid microbial degradation of bentazone (Hedegaard et al., **III**).

8.5 Bentazone degradation by methanotrophic biomass from aeration tanks

Methanotrophs produce large amounts of extracellular polymeric substance (EPS) to establish biofilms slime (Hilger et al., 1999; Hou et al., 1979). Hence, to avoid clogging, water utilities aim at avoiding growth of methanotrophs in the rapid sand filters. Thus, an efficient aeration system is essential to strip-off methane, before the rapid sand filters. Methane concentrations are therefore higher in the aeration tanks than in the rapid sand filters, and likewise is the growth potential of methanotrophs. We imagined that methanotrophs could be grown in the aeration tanks under high methane concentrations, and subsequently transported with the water to the rapid sand filters. In the rapid sand filters the methane concentration is lower or even absent, however, methanotrophs are a part of the core taxa (Albers et al., 2015a; Gülay et al., 2014) and the methane oxidation in filter sand confirmed their activity (Figure 16a and b) (Hedegaard et al., **V**). If the bentazone degradation in rapid sand filters is associated with methanotrophs grown in the aeration system, the bentazone degradation potential in biomass from the aeration tanks would be expected to be similar to the degradation in filter sand.

Thus, biomass from the aeration tanks at Ullerup and Stenholt waterworks was investigated for its bentazone degradation potential, and the association with methane oxidation. In absence of methane, no removal of bentazone was detected (21 days) (Hedegaard et al., **V**). Then methane was added to the microcosms with biomass from Stenholt, and methane consumption started (Figure 18). Along with the methane consumption, started the bentazone removal (Figure 18) (Hedegaard et al., **V**). No removal of ^{14}C-bentazone and no $^{14}CO_2$-production showed that the biomass most likely only performed the initial hydroxylation of bentazone, leading to OH-bentazone transformation products in the water phase, as observed in the methanotrophic enrichment culture (Hedegaard et al., **II** and **V**). Hence, bentazone biodegradation was clearly connected with the methane oxidation by the biomass from waterworks treating methane-rich groundwater (Hedegaard et al., **V**).

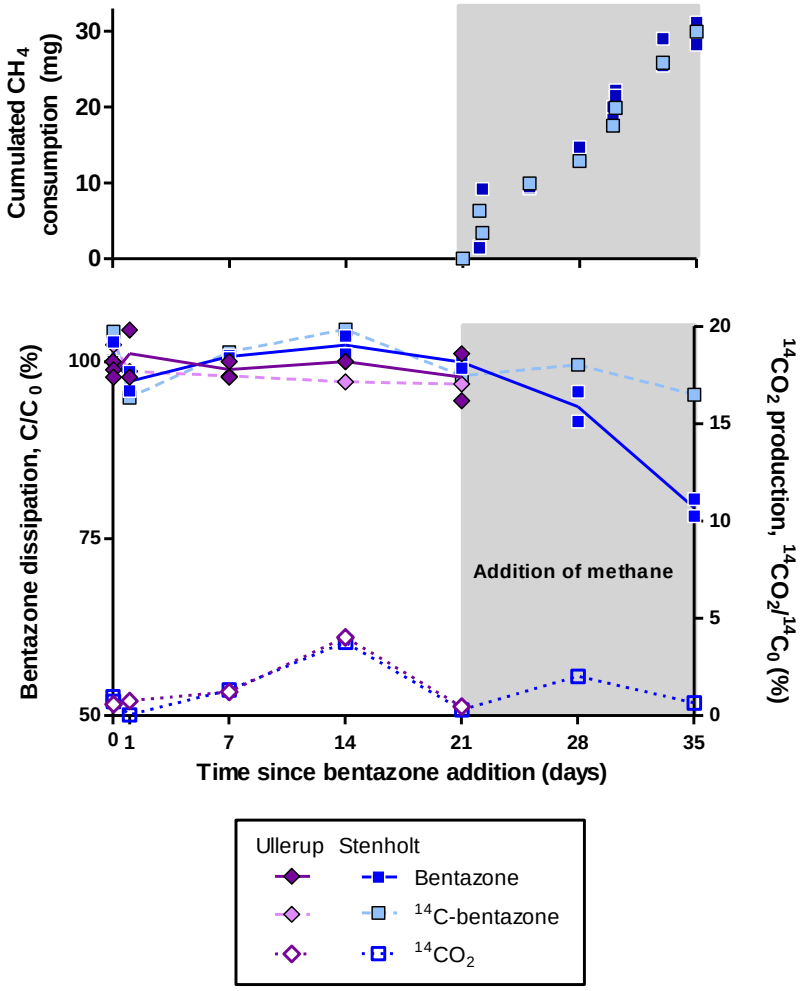


Figure 18. Bentazone removal by biomass from aeration tanks. Microcosms with 5 mL biomass from aeration tanks at Ullerup and Stenholt waterworks, 100 mL water and bentazone (1 mg/L, duplicates) or ^{14}C-carbonyl-bentazone (1 µg/L, single microcosm). Top) Cumulated methane consumption in microcosms from Stenholt. Bottom) ^{14}C-bentazone removal in microcosms (left y-axis) and produced $^{14}CO_2$ from mineralization (right y-axis), given as percentage of the initial concentration (C/C_0 or $^{14}CO_2/^{14}C_0$) (Sykyta and Milanovic, 2017; Hedegaard et al., **V**).

The similar bentazone degradation in biomass from aeration tanks and in filter sand indicated that the full-scale waterworks operates like a sequential reactor system, where methanotrophs were grown in the aeration tanks, and subsequently transported to the rapid sand filters (Hedegaard et al., **V**). Further studies should investigate whether methanotrophs in the rapid sand filters originate from the aeration tanks, and why presence of methane is essential for bentazone degradation by biomass from aeration tanks, while it does not influence the degradation in filter sand. Investigations on the methanotrophic community in the aeration tanks and in the rapid sand filters, could illuminate this process.

8.6 Biotransformation of bentazone in water treatment

The bentazone degradation pathways in filter sand showed that initial hydroxylation reactions were important for the biotransformation. Inhibition experiments with acetylene, showed that the major bentazone biotransformation was due to activity of monooxygenases. However, acetylene inhibits both the activity of ammonium monooxygenases (AMO) and methane monooxygenases (MMO) (Bédard and Knowles, 1989; Lontoh et al., 2000), which are both present in rapid sand filters (Albers et al., 2015a; Gülay et al., 2016). The bentazone biotransformation in filter sand correlated significantly with methane concentration, while there was no correlation with manganese, iron and ammonium concentration. In the biomass from the waterworks aeration tanks, bentazone removal only occurred under presence of methane. Altogether, these results showed that the major fraction of bentazone biotransformation was connected with methane oxidation in biological water treatment processes (Figure 19) (Hedegaard et al., **V**).

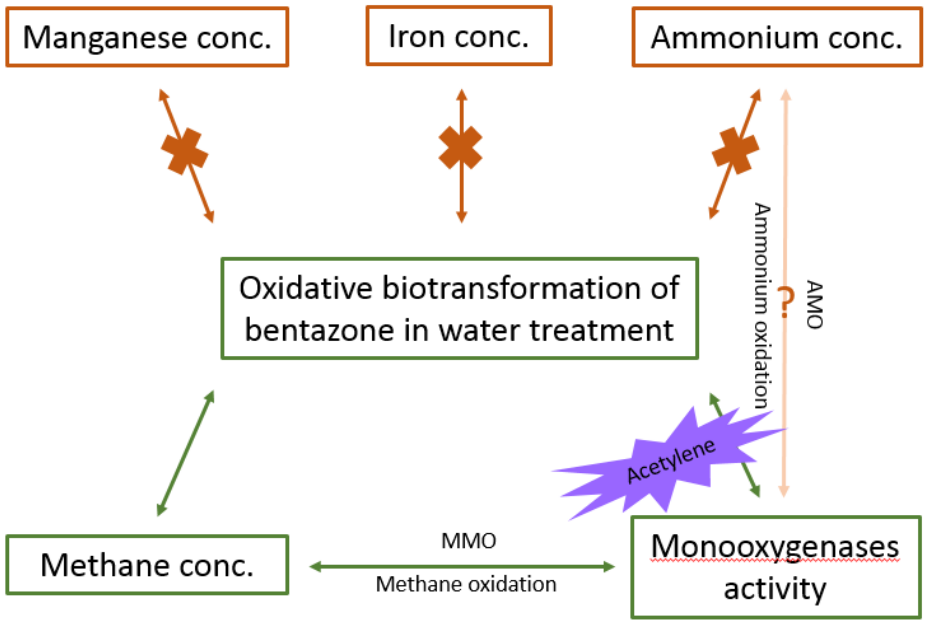


Figure 19 Association of bentazone biotransformation with different parameter in drinking water treatment. The major fraction of bentazone biotransformation was inhibited in the presence of acetylene, and was thus associated with activity of monooxygenases, such as methane monooxygenases (MMO) and ammonium monooxygenases (AMO). The methane oxidation stopped in the presence of acetylene, while the effect of acetylene on the ammonium oxidation was not measured. However, the bentazone did not correlate with the concentration of iron, manganese and ammonium, while it correlated significantly with the methane concentration (Hedegaard et al., **V**).

Summary: *Section 8 - Methanotrophic bentazone removal at waterworks*

- Presence of methane and bentazone in waterworks wells
  - Bentazone is detected significantly less frequently in waterworks wells with methane than in wells without methane in Denmark.
- Bentazone degradation in filter sand
  - The biological bentazone removal in filter sand from 14 waterworks correlated significantly with the maximum methane concentration in the raw water, and did not correlate with other water quality parameters, such as ammonium concentration.
  - High copper concentration, at a waterworks with high methane concentrations, was suggested to inhibit the expression of sMMO and thus inhibit the bentazone degradation.
  - The presence of methane did not affect bentazone removal in filter sand.
  - Acetylene inhibited methane consumption and removal of bentazone in the filter sand.
  - Three main biotransformation pathways were identified for bentazone in rapid sand filter. Initial hydroxylation reactions were essential for two of the degradation pathways.
  - One degradation pathway was initiated by *N*-methylation of bentazone, and was unlikely associated with methanotrophic activity, but similar to degradation in soils. A minor bentazone removal in presence of acetylene also indicated a non-methanotrophic removal.
- Bentazone degradation in biomass from aeration tanks
  - Methanotrophic biomass from the aeration tanks were capable of degrading bentazone, but only in presence of methane.
- Technical system
  - Further studies should investigate whether bentazone degradation in the filter sand was performed by methanotrophs, which were transported from the aeration tanks to the filters.

9 Methanotrophic transformation yields

To compare the methanotrophic co-metabolic degradation of bentazone (BTZ) across different media, e.g. filter sand, the transformation yield, $T_{y,TC/CH4}$, was calculated. The transformation yield expresses the removal rate of the trace contaminant (TC), in this case bentazone, over the methane removal rate (Anderson and Mccarty, 1997a). Removal rates were estimated by linear regression models (removed mass of bentazone or methane per time). At similar concentrations of bentazone (0.7-0.9 mg/L) and methane (approx. 5 mg/L), the transformation yields were within the range 4.2×10^{-5}-17×10^{-5} $mole_{BTZ}/mole_{CH4}$ for the methanotrophic enrichment culture, filter sand from two different waterworks and biomass from aeration tanks (Table 5) (Hedegaard et al., **II** and **V**). The similar transformation yields in all media strongly indicated that the same mechanism governed bentazone removal. The transformation yields were in the low range of values reported for chlorinated aliphatic hydrocarbons (2.2×10^{-4} to 6.3×10^{-1} $mole_{TC}/mole_{CH4}$) (Table 6). However, the ratio $C_{BTZ/CH4}$, between the secondary substrate, bentazone, and the primary substrate, methane, was also low (9.6×10^{-3}-13×10^{-3} $mole_{BTZ}/mole_{CH4}$) compared to reported values: 1.1×10^{-2} to 6.5 $mole_{TC}/mole_{CH4}$ (Table 6) (Hedegaard et al., **II**). The large difference in the relative abundance of primary and secondary substrates makes the comparison of the transformation yields between different studies irrelevant (Hedegaard et al., **II**).

To establish a metric independent of the relative concentrations of the substrates, Hedegaard et al. (**II**) suggested to normalize the transformation yield with respect to the concentration ratio between secondary and primary substrate, obtaining the normalized substrate preference:

$$SP_{CH4/TC} = T_{y,TC/CH4}^{-1} / C_{TC/CH4}^{-1} = T_{y,CH4/TC} / C_{CH4/TC}$$

Where $SP_{CH4/TC}$ is the normalized substrate preference, $T_{y,TC/CH4}$ is the transformation yield and $C_{TC/CH4}$ is the substrate concentration ratio. Observed normalized substrate preferences, $SP_{CH4/TC}$, calculated from literature ranged from 3 to 400 (Table 6) (Hedegaard et al., **II**). The $SP_{CH4/BTZ}$ was within this range (58-319) (Table 5) (Hedegaard et al., **II** and **V**). Hence, in even presence of bentazone and methane-molecules, bentazone would, at maximum, be oxidized in 1 out of 58 incidences. Similar magnitude in the preference of MMO for oxidizing methane over other trace contaminants, indicated that the removal mechanism of bentazone was similar to co-metabolic degradation of other trace contaminants by MMO (Hedegaard et al., **II**).

Table 5. Removal rates of bentazone and methane, transformation yield and normalised substrate preferences by methanotrophic enrichment culture, filter sand and biomass from aeration tanks. The consumption rates are derived from linear regression models (n refer to the number of data points). The transformation yield, $T_{y,BTZ/CH4}$, expresses the removal rate of bentazone (BTZ) over methane (CH_4). The normalized substrate preference, *SP*, is the transformation yield, normalized to the concentration ratio, $C_{TC/CH4}$ (Hedegaard et al., **II** and **V**)

		Acetylene	Mass	Time (days)	r_{BTZ} ($nmole_{BTZ}$/h/microcosm)	r_{CH4} ($\mu mole_{CH4}$/h/microcosm)	$T_{y,BTZ/CH4}$ ($mole_{BTZ}/mole_{CH4}$)	$C_{BTZ/CH4}$ ($mole_{BTZ}/mole_{CH4}$)	***SP*** **(CH_4/BTZ)**	Reference
Enrichment culture from SPII	Exp. 1	-	20 g	1	5.39±1.26 (r^2=0.65), n=12	39.4±4.70 (r^2=0.88), n=12	14×10^{-5}	9.6×10^{-3}	**70**	Hedegaard et al., **II**
		(+)	20 g	1	2.90±1.08 (r^2=0.42), n=12	17.6±5.60 (r^2=0.58), n=8	17×10^{-5}	9.6×10^{-3}	**58**	
	Exp. 2	-	10 g	15	0.37±0.05 (r^2=0.77), n=21	6.11±0.47 (r^2=0.81), n=42	6.1×10^{-5}	9.6×10^{-3}	**158**	Hedegaard et al., **II**
		+	10 g	15	0.04±0.04 (r^2=0.06), n=20	ND (r^2=0.12), n=45	NC			
Filter sand	SPII	-	100 g	13	0.66±0.04 (r^2=0.96), n=12	4.35±0.27 (r^2=0.91), n=28	9.4×10^{-5}	13×10^{-3}	**142**	Hedegaard et al., **V**
		+	100 g	13	0.25±0.04 (r^2=0.79), n=12	0.82±0.35 (r^2=0.21), n=22	NC			
	Stenholt	-	100 g	11	0.87±0.04 (r^2=0.98), n=10	15.6±0.60 (r^2=0.98,) n=30	4.2×10^{-5}	13×10^{-3}	**319**	Hedegaard et al., **V**
		+	100 g	11	0.22±0.03 (r^2=0.85), n=10	0.71±0.39 (r^2=0.15), n=20	NC			
Aeration tank	Stenholt	-	5 mL	11	0.26±0.04 (r^2=0.92), n=6	5.5±0.3 (r^2=0.97), n=18	4.6×10^{-5}	13×10^{-3}	**288**	Hedegaard et al., **V**

NC – not calculated since methane consumption was inhibited

Table 6. Comparison of normalized substrate preferences. Data from the present study compared to reported data (see reference). The comparison is based on maximum measured transformation yields, T_y, in absence of formate. T_y and the maximum aqueous concentration of methane (CH_4) and trace contaminant (TC) for cultures expressing sMMO and pMMO is given as in Anderson and Mccarty (1997). The normalized substrate preference, *SP*, is the transformation yield, normalized to the concentration ratio, $C_{TC/CH4}$ (Hedegaard et al., **II**).

Culture	Trace contaminant	Max. transformation yield	Max. aqueous conc. of		Conc. ratio	Normalized substrate preference	
		T_y (r_{TC}/r_{CH4})	CH_4	Trace contaminant	$C_{TC/CH4}$[a]	$SP_{CH4/TC}$[a] ($T_{y,CH4/TC}/C_{CH4/TC}$)	Reference
		($mole_{TC}/mole_{CH4}$)	(μM)	(μM)	($mole_{TC}/mole_{CH4}$)	-	
Mixed culture	TCE	4.9×10^{-3}	349	43	1.2×10^{-1}	**25**	Smith and McCarty (1997)
Pure cultures	TCE	5.3×10^{-3}	75	150	2.0	**377**	Fennell *et al.* (1993)
	TCE	7.5×10^{-3}	60	150	2.5	**333**	Phelps *et al.* (1990)
	VC	6.6×10^{-3}	6.3	2.2	3.5×10^{-1}	**53**	Nelson and Jewell (1993)
	TCE	1.9×10^{-3}	50	13	2.6×10^{-1}	**137**	Anderson and Mccarty (1997b)
	TCE	4.1×10^{-3}	4.7	7	1.5	**363**	Arvin (1991)
	1,1-DCE	2.2×10^{-4}	50	0.56	1.1×10^{-2}	**51**	Anderson and Mccarty (1997b)
	c-DCE	5.8×10^{-2}	30	86	2.9	**49**	Anderson and Mccarty (1997b)
	c-DCE	2.5×10^{-2}	4.7	28	6.0	**238**	Arvin (1991)
	t-DCE	5.7×10^{-1}	30	160	5.3	**9**	Anderson and Mccarty (1997b)
	t-DCE	3.9×10^{-2}	4.7	0.6	1.3×10^{-1}	**3**	Arvin (1991)
	t-DCE	6.3×10^{-2}	40	100	2.5	**40**	Janssen *et al.* (1988)
	t-DCE	6.3×10^{-1}	3.1	20	6.5	**10**	Anderson and Mccarty (1997a)
	VC	2.6×10^{-1}	205	208	1.0	**4**	Dolan and McCarty (1995)
	VC	2.0×10^{-1}	30	17	5.7×10^{-1}	**3**	Anderson and Mccarty (1997b)

[a] Calculated from data in given reference

Summary: ***Section 9 - Methanotrophic transformation yields of bentazone***

- The transformation yields ($T_y=r_{BTZ}/r_{CH4}$) of the methanotrophic enrichment culture, filter sand from two waterworks and biomass from aeration tanks were within the range 4.2×10^{-5}-17×10^{-5} $mole_{BTZ}/mole_{CH4}$.
- The similar transformation yields strongly indicated the same governing bentazone removal mechanism in the different media.
- The normalized substrate preference, $SP_{CH4/TC}$, expressed the preference of methane monooxygenases, MMO, for methane over trace contaminants.
- Similar magnitude in the normalized substrate preference of MMO, indicated that the removal mechanism of bentazone was similar to co-metabolic degradation of other trace contaminants by MMO.

10 Strategy for co-metabolic pesticide degradation

Bentazone was detected significantly less frequently in methane-rich groundwater compared to groundwater without methane. Thus, bentazone contaminations are naturally not a problem at waterworks treating methane-rich groundwater. Applying methanotrophs as treatment technology should therefore be design to fit waterworks without methane in the abstracted water.

In presence of methane, pesticide removal could not be detected during investigations with methanotrophic column reactors (Papadopoulou et al., **IV**). Due to competitive inhibition between the primary and secondary substrate, technical systems like the column reactors, encompass a conflict between providing sufficient methane to support growth, and thus gain sufficient quantities of MMO to degrade the secondary substrate, while balancing this to the inhibitory effects of the presence of methane (Jiang et al., 2010; Semprini and McCarty, 1992; Sullivan et al., 1998). However, this conflict has been partially managed by for example using sequential systems, where growth and co-metabolic biodegradation takes place in different reactors (Jiang et al., 2010; Smith and McCarty, 1997). It was suggested that the full scale waterworks operates like such a sequential reactor system, where methanotrophs are grown at high methane concentrations in the aeration system, and subsequently transported to the rapid sand filters, where they can perform co-metabolic biodegradation in absence or low concentrations of methane.

In contrast to reported inhibitory effects on the co-metabolic degradation by the presence of methane (Jiang et al., 2010; Sullivan et al., 1998), presence of methane was necessary to obtain bentazone degradation in biomass from the aeration tanks. Similarly, presence of methane stimulated bentazone degradation by the methanotrophic culture enriched from rapid sand filters, while it did not affect bentazone degradation in filter sand. Thus, the presence of methane did not inhibit the bentazone degradation.

Hence, it is suggested that co-metabolic pesticide degradation can be implemented in full-scale waterworks by addition of methane to the raw water. The methane content should be sufficient to sustain growth of methanotrophs in the aeration system (bioaugmentation with desired methanotrophic species might be necessary), but should not exceed the amount, which will be stripped-off during the aeration process. This method would of cause only be applicable to manage pesticides which can be co-metabolically degraded by methanotrophs,

e.g. bentazone. A challenge for the implementation of this technology is to ensure the optimal composition of the methanotrophic community, which can be affected by for example the copper concentration (Hedegaard et al., **V**).

Thus further studies should investigate whether 1) co-metabolic pesticide degradation can be implemented at full-scale waterworks by adding methane to the raw water, 2) existing full-scale waterworks can operate as a sequential methanotrophic reactor systems, 3) which pesticides could be managed in this system by co-metabolic degradation, and 4) the optimal operation condition including investigation of the methanotrophic community, copper and methane concentrations.

Summary: ***Section 10 – Strategy for co-metabolic bentazone degradation***

- Methanotrophic co-metabolic pesticide degradation should be designed to fit waterworks without methane in the abstracted water
- Further studies should investigate:
  - Whether co-metabolic pesticide degradation can be implemented at full-scale waterworks by adding methane to the raw water
  - Whether full-scale waterworks operate as a sequential methanotrophic reactor systems, where methanotrophs are grown in aeration tanks and transported to the rapid sand filters.
  - Which pesticides can be managed a waterworks by methanotrophic degradation
  - The optimal operation condition including investigation of the methanotrophic community, copper and methane concentrations

11 Conclusions

In this PhD-thesis I have investigated microbial pesticide degradation potentials and processes at waterworks treating groundwater. The main conclusions are:

A full-scale rapid sand filter was able to remove a MCPP contamination from drinking water. There was a potential for removing several pesticides (e.g. phenoxy acids, glyphosate and bentazone) in filter sand from rapid sand filters at three waterworks. Removal of the investigated pesticides began immediately, and thus the degrading organisms were already present in the full-scale filters. The largest biological removal was observed in filter sands from waterworks characterised by having high concentrations of methane in the raw water.

Methanotrophs contributed to the degradation of phenoxy acids in a methanotrophic enrichment culture. However, the omnipresent degradation of MCPP in filter sand from 10 different waterworks, receiving groundwater with varying concentrations of methane, demonstrated that degradation of phenoxy acids in rapid sand filters was not associated with methane oxidation. Other studies have shown that sand filters are easily enriched with specific phenoxy acid degraders upon exposure to these pesticides. Based on the present investigations and literature, it was suggested that phenoxy acid degradation in rapid sand filters is due to primary metabolism, and that degradation might be stimulated by enriching naturally occurring specific degraders by exposing filters to phenoxy acid contaminated groundwater.

A suite of evidence showed that bentazone was co-metabolically transformed by a methanotrophic enrichment culture. Hence, the presence of methane stimulated the bentazone removal rate and increased the formation of hydroxy-bentazone transformation products. Inhibition of the methane oxidation by acetylene halted bentazone removal, while high concentrations of bentazone inhibited the methane consumption.

Methanotrophic bentazone removal was investigated in the water treatment systems. Bentazone was detected significantly less frequently in waterworks wells with methane, than in wells without methane, indicating that methanotrophs in real environmental systems protect methane-rich groundwater against bentazone contaminations. The biological bentazone removal in filter sand from 14 waterworks correlated significantly with the methane-concentration in the raw water and did not correlate with other water quality parameters e.g. ammonium concentration. Additionally, acetylene inhibited the methane-

consumption and removal of bentazone in the filter sand. Because acetylene inhibited bentazone removal, showing the involvement of monooxygenases in the degradation, and bentazone removal only correlated with the methane concentration, and not with e.g. ammonium concentration, it was suggested, that bentazone removal in filter sand was connected with the methane oxidation.

Three main biotransformation pathways of bentazone were identified in filter sand. These clearly showed the importance of hydroxylation reactions during bentazone degradation, but also that other heterotrophs in filter sand degraded bentazone further. *N*-methylation initiated one of the degradation pathways, and was unlikely associated with methanotrophic activity, but similar to degradation in soils. A minor bentazone removal in microcosms, where methane oxidation was inhibited by acetylene also showed a contribution by non-methanotrophs to the degradation of bentazone in filter sand. Hence, in real filter sand *both* methanotrophs and other heterotrophs contributed to bentazone degradation, and this led to a partially mineralization.

Methanotrophic biomass from the aeration tanks degraded bentazone, and this degradation depended on methane oxidation. Transformation yields of bentazone versus methane were within the same range for the methanotrophic enrichment culture, filter sand and biomass from an aeration tank. Thus, the same process governed bentazone removal in the different media, and that this process was connected with the methane oxidation. It was suggested that the full-scale waterworks operates like a sequential system, where methanotrophs are grown in the aeration tanks (under high methane concentrations) and transported to the rapid sand filters, where they can perform co-metabolic biodegradation of e.g. bentazone. Further studies should investigate whether bentazone removal can be initiated by stimulating growth of methanotrophs in water treatment systems.

Overall, this thesis show a substantial potential for biological degradation of pesticides in rapid sand filters. The omnipresent phenoxy acid degradation potential in filter sand was probably due to primary metabolism, while bentazone degradation was connected with methane oxidation. Thus, different pesticides require different strategies for stimulation of degradation. Based on the present investigations and literature, it was suggested that phenoxy acid degradation can be stimulated by enrichment of naturally occurring degraders in filter sand, and that bentazone degradation can be stimulated by stimulating growth of methanotrophs in the water treatment.

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II **Hedegaard, M. J.**, Deliniere, H., Prasse, C., Dechesne, A., Smets, B. F., Albrechtsen, H.-J., 2018. Evidence of co-metabolic bentazone transformation by methanotrophic enrichment from a groundwater-fed rapid sand filter. *Water Research,* Vol. 129, pp 105-114.

III **Hedegaard, M. J.,** Prasse, C., Albrechtsen, H.-J. Degradation pathways of the herbicide bentazone in filter sand used for drinking water treatment. *Submitted*

IV Papadopoulou, A., **Hedegaard, M. J.,** Dechesne, A., Albrechtsen, H.-J., Musovic, S., Smets, B. F. Methanotrophic contribution to phenoxy acids degradation in cultures enriched from a groundwater-fed rapid sand filter. *Manuscript*

V **Hedegaard, M. J.**, Sykyta, M. A. M., Milanovic, N., Lee, C. O., Boe-Hansen, R., Albrechtsen, H.-J. Importance of methane oxidation for microbial degradation potential of the herbicide bentazone in drinking water production. *Draft manuscript*

In this online version of the thesis, **paper I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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